The role of dendritic cells in the pathogenesis of *Staphylococcus aureus* infection

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1. Abstract

Dendritic cells (DCs) play an essential role in orchestrating and regulating the immune response against many pathogens. In this dissertation, the importance of DCs in host defense against the clinical relevant bacterium *Staphylococcus aureus* (*S. aureus*) was investigated.

Immunofluorescence live-cell imaging was used to demonstrate the high efficiency of DCs to phagocyte *S. aureus*. Bacterial uptake induced a fast maturation of DCs as shown by the up-regulation of the co-stimulatory molecules CD40, CD80, CD86, and MHC class II, as well as by the production of the inflammatory cytokines IL-6, IL-12, and TNFα. In addition, DCs were rapidly mobilized and recruited into infected tissue after intravenous inoculation of mice with *S. aureus*. CD11c-DTR transgenic mice were then used to evaluate the relevance of DCs for host defense against *S. aureus.* These transgenic mice express the diphtheria toxin (DT) receptor on the CD11c promoter region, which allows for selective depletion of DCs followings the intraperitoneal administration of DT. *In vivo* depletion of DCs resulted in accelerated mortality and significantly increased bacterial loads in the lungs and kidneys of infected mice. Reconstitution of DC-depleted mice by adoptive transfer of DCs from BALB/c mice returned the capacity of these animals to control *S. aureus* infection. Furthermore, increase in DCs numbers in normal mice resulted in enhanced resistance to infection.

The beneficial effect afforded by DCs was not attributed to a direct contribution to bacterial killing. Interestingly, depletion of DCs resulted in a more rapid influx of primary polymorph nuclear neutrophils (PMNs) to the site of infection; a significant increase in the local production of CXC chemokines and increased survival of *S. aureus* within the recruited PMNs.

The results of this study provide compelling evidence that DCs are important regulators of the host immune response to *S. aureus* and of benefit for the host. These data may be of use in future studies for the development of new therapeutic strategies for the treatment of *S. aureus* infections.

2. Introduction

2.1 *Staphylococcus aureus*

2.1.1 Microbial characterization of *Staphylococcus aureus*

The species *Staphylococcus aureus* (*S. aureus*) is the most prominent of the genus *Staphylococcus* in relation to human diseases [1, 2]. It was originally discovered by the surgeon Sir Alexander Ogston in pus from surgical abscesses in the year 1880 and described as:

> "Micrococcus, which, when limited in its extent and activity, causes acute suppurative inflammation (phlegmon), produces, when more extensive and intense in its action on the human system, the most virulent forms of septicæmia and pyæmia" [3, 4].

S. aureus is a gram-positive, non motile, non spore-forming facultative anaerobic, catalase and coagulase positive coccus that is able to induce ß-haemolysis. Traditionally, it was considered to be an extracellular pathogen [2, 5], but increasing evidence indicates that *S. aureus* might be a facultative intracellular pathogen [6-10]. Staphylococci appear in grape-like clusters. The species name "aureus", which means "golden" in Latin is due to the yellow-golden color of the colonies [11]. The color is imparted by carotenoid pigments that supports the bacteria in the prevention of oxidant killing by phagocytes [12]. Around 20% of the population is persistently and around 30% intermittently colonized by *S. aureus* in the nose or in the axilla region, groin areas, or gastrointestinal tract [13].

2.1.2 *S. aureus* virulence factors

The remarkable success of *S. aureus* as a pathogen is due to the expression of a wide array of virulence factors including a capsule as well as surface-exposed and secreted proteins [14-16]. A schematic representation of *S. aureus* virulence factors is depicted in Figure 1. In more detail, coagulase is an extracellular protein that reacts with prothrombin in the blood to form a complex called staphylothrombin. The protease activity of this

complex converts soluble fibrinogen to fibrin. This results in clotting of the blood that might protect the bacterium from phagocytic uptake by immune cells [17]. The clumping factor binds serum fibrin/fibrinogen and reduces the activity of phagocytic immune cells [18, 19]. Protein A is able to bind immunoglobulins through their Fc region thereby hampering Fc receptor-mediated opsonophagocytosis [20, 21]. Furthermore, protein A can induce T cell-independent proliferation and apoptosis of B cells [22]. The elastin-binding protein mediates adhesion of *S. aureus* to elastin-rich cell surfaces such as lungs, skin, or blood vessels [23], while the collagen-binding [24, 25] and the fibronectin-binding [24, 25] proteins support adhesion of *S. aureus* to connective tissue and extracellular matrix. Enterotoxin B and toxic-shock-syndrome-toxin 1 (TSST-1) are bacterial superantigens that bind MHC II complexes on antigen-presenting cells leading to immuno-suppression [26-28]. The α-toxin is the best-characterized and more potent cytotoxin of *S. aureus* [29]. It binds to cell surface receptors and lipid bilayers on host cells and form pores that eventually cause cell death [29-31].

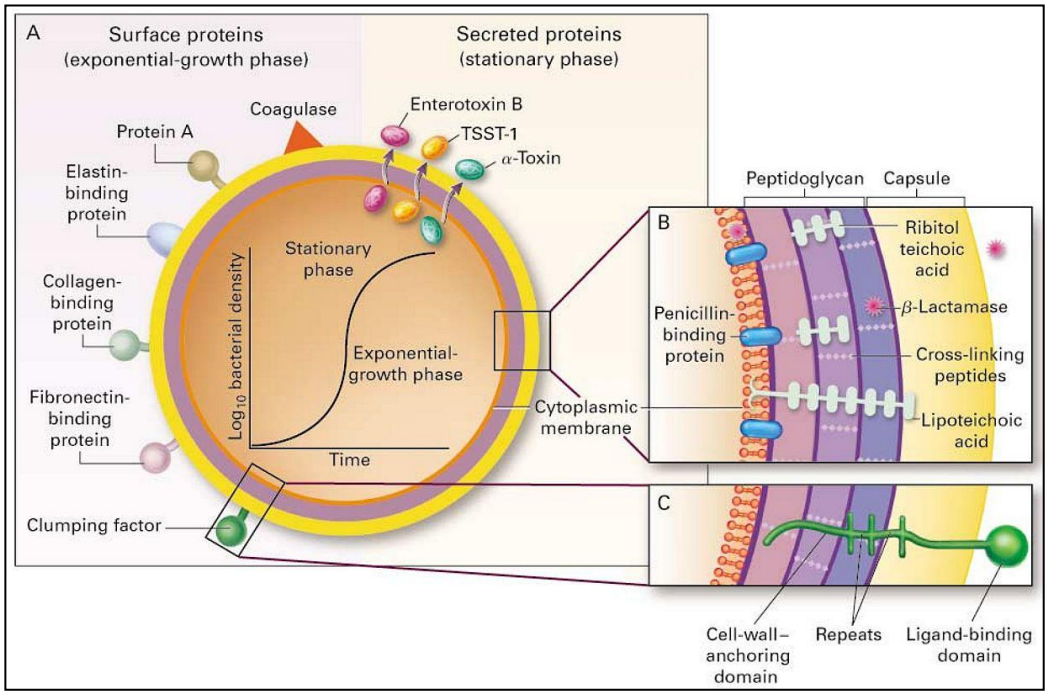

Figure 1: Schematic representation of *S. aureus* virulence factors. Surface and secreted proteins according to growth phase are shown in panel A. Cross sections of cell envelope are shown in panels B and C. Figure taken from Lowy, 1998 [2].

2.1.3 Adhesion and invasion of *S. aureus*

Adherence of *S. aureus* to the host extracellular matrix is the initial step in the infection process and is mediated by bacterial surface adhesins, known as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) [32]. MSCRAMMs bind to extracellular matrix proteins such as fibrinogen (ClfA, ClfB), fibronectin (FnbpA, FnbpB), and collagen (Cna) [33]. Certain MSCRAMMs, such as ClfA and SdrC are found in virtually all clinical *S. aureus* strains, whereas others, such as Cna and SdrD, are present only in a subset of strains [34]. In addition to mediate the bacterium attachment to the extracellular matrix, certain MSCRAMMs are able to mediate the internalization of *S. aureus* within eukaryotic cells. *S. aureus* is generally thought to be an extracellular pathogen, but it can be internalized by a variety of cell types *in vitro* including fibroblasts, osteoblasts, keratinocytes, and endothelial cells [35]. This may be an important process in systemic spread of infection, escape from antibiotic pressure and evasion of the host immune system [36]. The dynamic process of *S. aureus* attachment and invasion is illustrated in Figure 2.

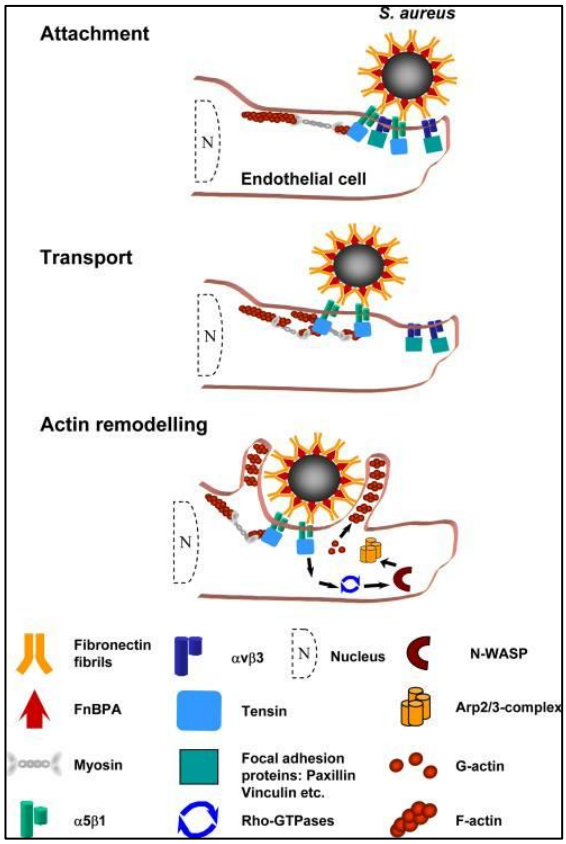

Figure 2: Attachment and invasion of *S. aureus* in endothelial cells.
Figure taken from Schröder *et al.*, 2006 [37].

The schematic representation in Figure 2 shows *S. aureus* attached to α5β1-integrins on the surface of endothelial cells via fibronectin fibrils that are bound to the fibronectin-binding protein FnBPA (attachment). The host cell integrins are associated with myosin bound tensins, and by contraction of the myosin, the bacteria are transported towards the cell center (transport). The uptake is performed by tensin-mediated actin reorganization (actin remodeling).

2.1.4 *S. aureus* infections

S. aureus can cause a wide variety of infections including skin and soft-tissue infections such as folliculitis, impetigo, furuncles, carbuncles, and cellulites [2]. If the bacterium is able to enter the bloodstream, it can cause live-threatening invasive infections such as bacteremia, infective endocarditis, osteomyelitis, or sepsis [38, 39]. Other infections caused by *S. aureus* include catheter-related infections, joint infections, pulmonary infections, and central nervous system infections [39]. *S. aureus* is associated with an increasing number of both community-acquired and hospital-acquired cases of pneumonia. Recent studies have reported that *S. aureus* comprises more than 25% of community-acquired and more than 40% of hospital-acquired pneumonia cases [40]. A most severe outcome of *S. aureus* bacteremia is the development of sepsis. Sepsis is described as an extensive clinical syndrome that is due to an overwhelming response of the immune system to an infection progress, followed by immunosuppression [41, 42]. Whereas the most important mediator of gram negative-associated sepsis is the lipopolysaccharide (LPS), in gram-positive bacteria the cell wall components peptidoglycan and lipoteichoic acid play a dominant role. In addition to these components, some strains of *S. aureus* produce diverse exotoxins like TSST-1, which act as superantigens and are therefore able to induce T lymphocyte activation followed by a massive release of pro-inflammatory cytokines [43].

2.1.5 Antibiotic resistance in *S. aureus*

Treatment of *S. aureus* infections can be difficult since many strains have developed resistance to several antibiotics. Penicillin was the first antibiotic introduced in the clinic to treat *S. aureus* infections. The mode of action of penicillin consists in the inhibition of peptidoglycan synthesis on the bacterial cell wall [44, 45]. Shortly after its introduction, *S. aureus* strains developed resistance against beta-lactam antibiotics by the acquisition of the enzyme beta-lactamase that hydrolytically destroys the beta-lactam ring [46]. Methicillin, introduced in 1961, was the first of the semisynthetic penicillinase-resistant penicillins due to a different steric orientation of the beta-lactam ring. Unfortunately, *S. aureus* rapidly developed resistance against methicillin and other semi-synthetic penicillins [47-49]. Resistance to methicillin is mediated by the acquisition of the mecA gene, which codes for the penicillin-binding protein PBP2a [50]. Methicillin-resistant strains of *S. aureus* are commonly abbreviated as MRSA. In the USA, the percentage of MRSA strains rose from 11% to 59.5% between the years 1998 – 2003 [51]. In Europe, the epidemiology of MRSA varies widely depending on the country, ranging from 0.6% in Sweden to an average of 41% in Belgium, Greece, Ireland, and the United Kingdom [52]. Vancomycin has been the most reliable therapeutic agent against infections caused by MRSA strains. This glycopeptide antibiotic was introduced in the year 1958 for the treatment of gram positive bacterial infections [53]. However, in 1996 the first MRSA resistant to vancomycin was isolated from a Japanese patient [54, 55]. Subsequent isolation of several vancomycin resistant *S. aureus* (VRSA) strains from different countries has confirmed that the emergence of vancomycin resistance in *S. aureus* is a global issue.

2.2 The host immune response to *S. aureus*

With the exception of diseases caused by specific toxins, the pathogenicity of *S. aureus* is mediated by a variety of exoenzymes, adhesins, immunomodulating proteins, and the presence of cell wall compounds [56]. These virulence factors in combination allow the bacterium to infect virtually any

host tissue [57]. Recognition of *S. aureus* components by innate immune cells is the first step of host defense. The toll-like receptors (TLRs) constitute a family of receptors, which distinguish pathogen-associated molecular patterns such as LPS, peptidoglycan, and lipoprotein [44, 58]. Triggering of TLR signaling after pathogens recognition leads to the activation of transcription factors that in turn induce transcription of genes coding for a variety of proteins including cytokines and inflammatory mediators [59]. This results in massive invasion of immune cells to the site of infection. Virulence studies using knockout mice have shown that TLRs play a critical role in the innate immune response to staphylococcal infections since mice deficient in the expression of myeloid differentiation factor MyD88, a cytoplasmic adaptor molecule essential for the signaling of IL-1R/TLR family, are highly susceptible to this pathogen [60].

The staphylococcal protein A is a ligand for the TNF-α receptor 1, which is largely expressed on the airway epithelium. The interaction between protein A and the TNF-α-receptor 1 leads to an inflammatory response on epithelial cells and the recruitment of primary polymorph nuclear neutrophils (PMNs) [61].

An effective immune response against *S. aureus* involves PMN recruitment, which are required for bacterial clearance, and abscess formation [62-64]. The importance of PMNs in host defense against *S. aureus* infection has been demonstrated by recurrent *S. aureus* infections in patients with chronic granulomatous disease, who have a defect in the NADPH oxidase and respiratory burst [65]. The resulting substantial invasion of PMNs to the site of *S. aureus* infection is accompanied by central liquefaction necrosis and formation of peripheral fibrin walls in an effort to prevent microbial spread [66]. These structures are known as abscesses and they generally have a defined fibrinous wall surrounding a purulent focus of PMNs and bacteria.

The early events of the innate immune response culminate in the activation of the adaptive immune system, during which T and B cells capable of specific antigen recognition are generated.

2.3 Dendritic cells

2.3.1 Origin and subdivision of dendritic cells

Dendritic cells (DCs), discovered by Ralph M. Steinmann in the year 1973, represent a special type of leukocytes responsible for activation and control of both innate and adaptive immune responses [67]. In general, they develop in the bone marrow from myeloid precursors that also produce monocytes (see schematic representation in Figure 3). DCs are especially distributed in tissues that interface the external environment, such as the skin, the gut, and the lungs, where they can perform a sentinel function for incoming pathogens, and have the capacity to recruit and activate cells of the innate immune system [68-70]. After recognition of pathogens, DCs undergo a maturation process that leads to their migration to the T cells area of lymph nodes where antigen specific cells of the adaptive immune response can be primed. DCs are the only cell type capable of initiating adaptive immune responses by activating naïve T lymphocytes. Therefore, DCs serve as a major link between the innate and the adaptive immune response.

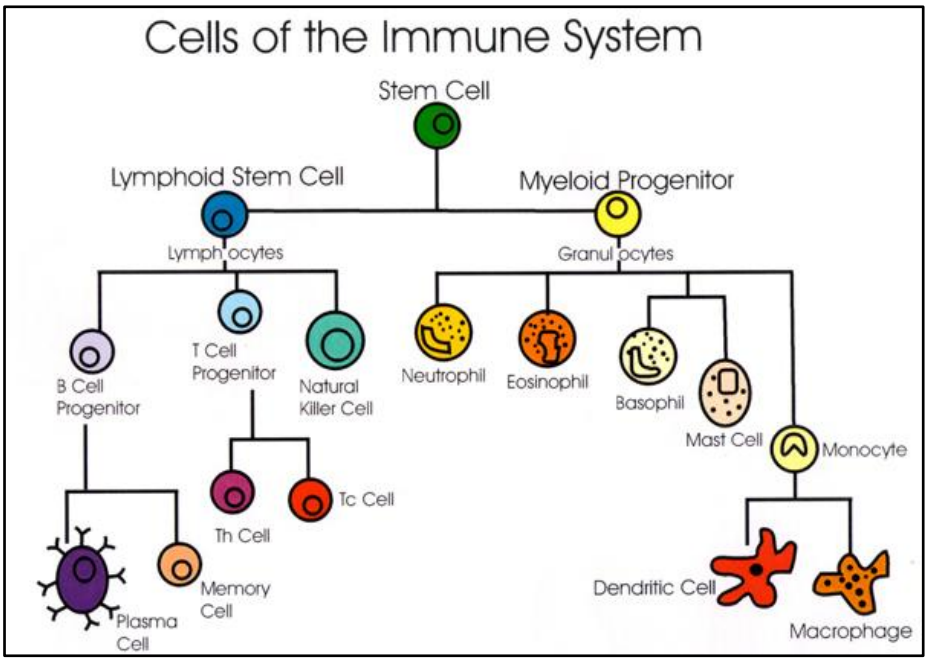

Figure 3: Schematic representation of the origin and differentiation of immune cells. Taken from Todar *et al.*, 2011 [71].

2.3.2 Maturation of DCs

DCs reside in an immature state in several tissues such as skin, pharynx, esophagus, or genitals. Furthermore, they are found in mucous membranes like respiratory and gastrointestinal tracts [72, 73]. Immature DCs have a radiating shape with their extensions ("dendrits") outstretched through the tight junctions of tissue epithelia cells, which increases their ability to catch antigens [74]. After recognition of pathogens through surface-exposed receptors, DCs increase their expression of the chemokine receptor CCR7, which enables them to migrate from the site of infection through the afferent lymphatic vessels to the T cell area of the draining secondary lymphatic organs (lymph nodes, Peyer-Plaques in the gut, tonsils, spleen, and appendix) [75, 76]. During migration, DCs undergo a maturation program that results in the up-regulation of co-stimulatory molecules such as CD40, CD80, and CD86, translocation of their MHC class II to the cell surface and secretion of inflammatory cytokines (see schematic representation in Figure 4) [77]. Furthermore, the morphology of the DCs also changes with maturation. These changes involve reduction of dendrits and development of membrane excrescences. The phagocytic capacity of DCs is also lost during the maturation process [44].

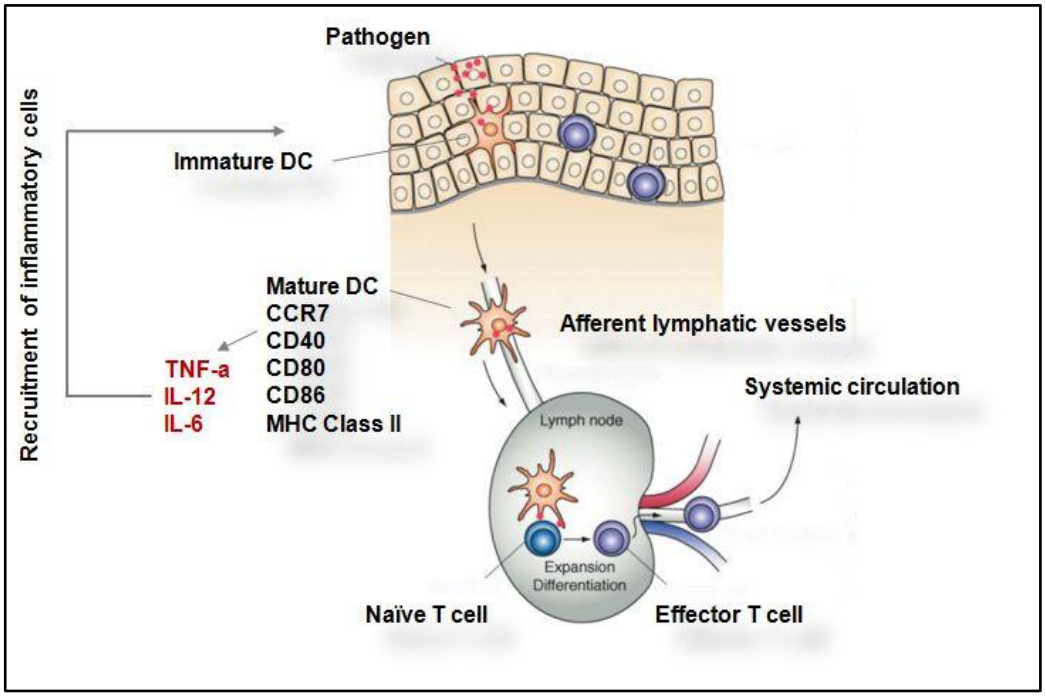

Figure 4: Schematic representation of maturation and migration of dendritic cells. Taken from [78], modified.

2.3.3 Activation of T lymphocytes

After reaching a lymphatic organ, DCs express a high amount of co-stimulatory molecules to activate the different types of T cells. By presenting the antigen via the MHC class I or II complexes and the interaction between their B7 molecules with the CD28 receptor on the T cells, the T cells become activated and leave the lymphatic organ via the efferent lymphatic vessels (see Figure 4 and Figure 5) [44].

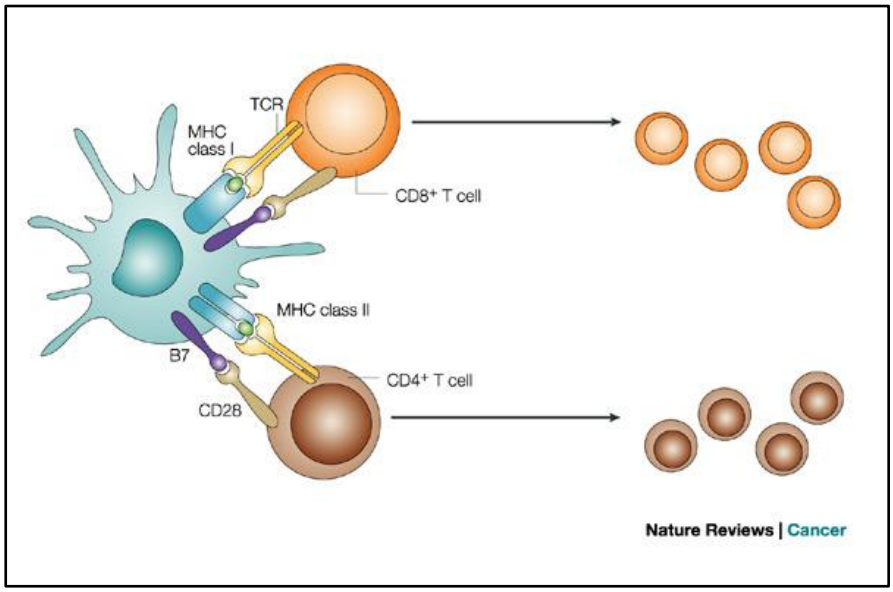

Figure 5: Activation of T lymphocytes by DCs. Taken from Gilboa *et al.*, 2004 [79].

2.3.4 DC subtypes

Different subtypes of DCs have been described according to the surface expression of specific markers and tissue distribution [80, 81]. In mice, immature conventional DCs are characterized by the expression of CD11c, low levels of the co-stimulatory molecules CD80 and CD86, and low levels of MHC class II. Interestingly, DCs can also express the T cell markers CD4 and CD8 as well as CD11b. The $CD8^-$ DCs reside mostly in the marginal zone of the lymph nodes while the $CD8^+$ are mainly located in the T cell area [81]. Another $CD4^-$ $CD8^-$ $CD11b^+$ DC population also expressing moderate levels of the scavenger receptor CD205 has been identified in all lymph nodes [82]. Finally, DCs in the skin, also known as

Langerhans cells, express high levels of Langerin [83]. In humans, DC subtypes are less characterized. Human DCs do not express CD8 and, in spleen and tonsils, DCs are positive for CD11b, CD11c, and CD4 [81].

2.3.5 DCs and their role in disease

Due to their excellent ability to present antigens and thereby activate and orchestrate the adaptive immune response, DCs play a pivotal role in the host defense against pathogens [77]. In this regard, several studies have demonstrated that DCs are required for the survival of mice suffering of polymicrobial sepsis [84]. Decreased counts of circulating DCs have been reported to correlate with disease severity and predicting a fatal outcome in patients affected by the septic shock syndrome [85]. Furthermore, transfer of bone-marrow derived DCs into the lungs of postseptic mice reversed immunosuppression and conferred protection against *Aspergillus* infection [86]. The importance of DCs has also been proved in non-septic situations. For example, DCs are an important inducer of T cell responses during *Cryptococcus neoformans* infections [87] and contribute to the host defense against *Streptococcus pyogenes* [88, 89]. Due to the importance of DCs for host defense, certain pathogens have developed mechanisms to avoid or even to use the DCs for their own benefit. Thus, DCs infected with the measles virus undergo maturation but are unable to induce T cell activation and show a high apoptosis rate [90, 91]. However, apoptotic measles virus-infected DCs are strong inducers of maturation of uninfected DCs, what may finally contribute to viral clearance [91]. By replicating and surviving within the DCs, *Mycobacteria* and *Salmonella sp.* can use this eukaryotic cell to disseminate within the host [92-95].

2.3.5 Pathogens strategies to evade DCs

As the DCs play a central role in the regulation of the immune response, many pathogens have developed strategies to prevent DC recognition. *Salmonella enterica* serovar Typhi is a motile bacterium that causes typhoid fever in humans. Their flagellin subunit FliC is an attractive target for DCs [96]. It has been shown, that the bacteria dramatically reduce the production of FliC as soon as they invaded into the gut cells, as motility is no longer required [97]. Therefore, by directing the DCs to induce a T cell response against an antigen that is no longer present, *Salmonella* evades the host immune response. *Yersinia enterocolitica* shows a more direct strategy to evade DCs. During infection, the bacteria are able to inject effector proteins like *Yersinia* outer proteins (Yops) into the host cells, which inhibits antigen presentation by the DCs [98]. Furthermore, Yops proteins induce DC apoptosis, and therefore hindering the appropriate stimulation of T cells due to the reduced number of DCs [99, 100]. Other pathogens such as *Helicobacter pylori* are able to inhibit the cytokine release of DCs by secreting a non-proteinaceous factor and thereby regulating the immune response to its favor [101]. *Streptococcus pneumoniae* produces haemolytic pneumolysin that inhibits the DC maturation, the induction of proinflammatory cytokines, and the activation of the inflammasome. If the bacteria are taken up by DCs, the intracellular production of pneumolysin induces caspase-dependent apoptosis [102]. In summary, the strategies developed by different pathogens to evade DCs are based upon their interference with the generation and survival of DCs, the antigen-presenting mechanisms, or the interference with T cell activation.

3. Aim of the work

In the last decades, *S. aureus* has been recognized as one of the most important leading cause of hospital- and community-acquired infections worldwide. As the management of *S. aureus* infections is a growing clinical challenge due to the widespread presence of multidrug-resistant *S. aureus* strains, new therapeutic options are therefore needed for the treatment of these infections in a more efficient way. This clinical challenge underscores the importance of a better understanding of the pathogenesis and cellular mechanisms underlying *S. aureus* infections. As DCs play a crucial role in protecting the host against pathogens, the aim of the current study was to investigate the role of DCs in the complex process of *S. aureus* invasive infection. For this purpose the interactions between *S. aureus* and DCs has been examined using *in vitro* as well as *in vivo* experimental systems. Live-cell imaging and immunofluorescence techniques have been applied to visualize the phagocytic process of *S. aureus* by DCs. In addition, CD11c-DTR transgenic mice, which allow the selective depletion of DCs *in vivo*, have been used to evaluate the relevance of DCs for the host defense against *S. aureus*.

4. Material and Methods

4.1 Bacterial strains

The *S. aureus* strain SH1000 was used in this study. SH1000 was developed in the year 2002 as a single-copy *rsbU* gene-complemented derivative of the wild-type strain 8325-4 [103]. *rsbU* encodes a positive regulator for sigma factor b activity, that is responsible for a general stress response [103]. Bacteria were grown to the Mid-Log phase at 37 ºC with shaking (150 rpm) in brain heart infusion (BHI)-medium, collected by centrifugation for 10 min at 4000 rpm, washed twice with sterile PBS and adjusted to 5 x 10^8 CFU/ml. For the inoculum preparation, the bacterial suspension was diluted with PBS to the required concentration and the number of viable bacteria was determined after serial diluting and plating onto agar containing 5% of sheep blood (Invitrogen, Karlsruhe, Germany). For live-cell imaging, a GFP-expressing SH1000 strain was used [104]. Bacteria were grown to the Mid-Log phase at 37°C with shaking (150 rpm) in brain heart infusion (BHI)-medium supplemented with 30 µg/ml of Chloramphenicol, collected by centrifugation for 10 min at 4000 rpm, washed twice with sterile PBS and adjusted to 5 x 10^8 CFU/ml. To generate heat-killed *S. aureus*, 10^9 bacteria were resuspended in 2 ml of PBS and incubated in a water bath for 2 h at 95°C.

4.2 Mice

BALB/c mice were purchased from Harlan-Winkelmann (Borchen, Germany), B6.FVB-Tg [Itgax-DTR/GFP] 57Lan/J transgenic mice [105] (referred in this study as CD11c-DTR) were obtained from Steffen Jung (The Weizmann Institute of Science, Rehovot, Israel) and backcrossed against a BALB/c background. These mice express a human diphtheria toxin (DT) receptor in the promoter region of CD11c. Mice harboring this transgene are transiently depleted of $CD11c^+$ populations upon intraperitoneal injection of diphtheria toxin (DT) (see Figure 6). DC depletion persists for approximately 2 days after which the cell population gradually recovers. For

systemic depletion of DCs, CD11c-DTR transgenic mice were injected intraperiotoneally (i.p.) with 4 ng/g body weight of DT (Sigma, Deisenhofen, Germany) in PBS 24 h prior to infection. CD11c-DTR injected i.p. with PBS were used as control. Ovalbumin specific mice B6.Cg-Tg (TcraTcrb) 425Cbn/J (referred in this study as OT-II) were purchased from Jackson Laboratory (Maine, USA). These mice express the mouse alpha-chain and beta-chain T cell receptor that pairs with the CD4 co-receptor. The T cell hybridoma clone is $CD4^+$, MHC class II-restricted, and is specific for the ovalbumin residue 323-339 peptides in the context of the MHC class II $I\text{-}A^2$ molecule [106].

Mice were housed in a specific pathogen-free animal facility at the Helmholtz Centre for Infection Research. All experiments were approved by the ethical board Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg in Germany, reference number 33.11.42502-04-024/08.

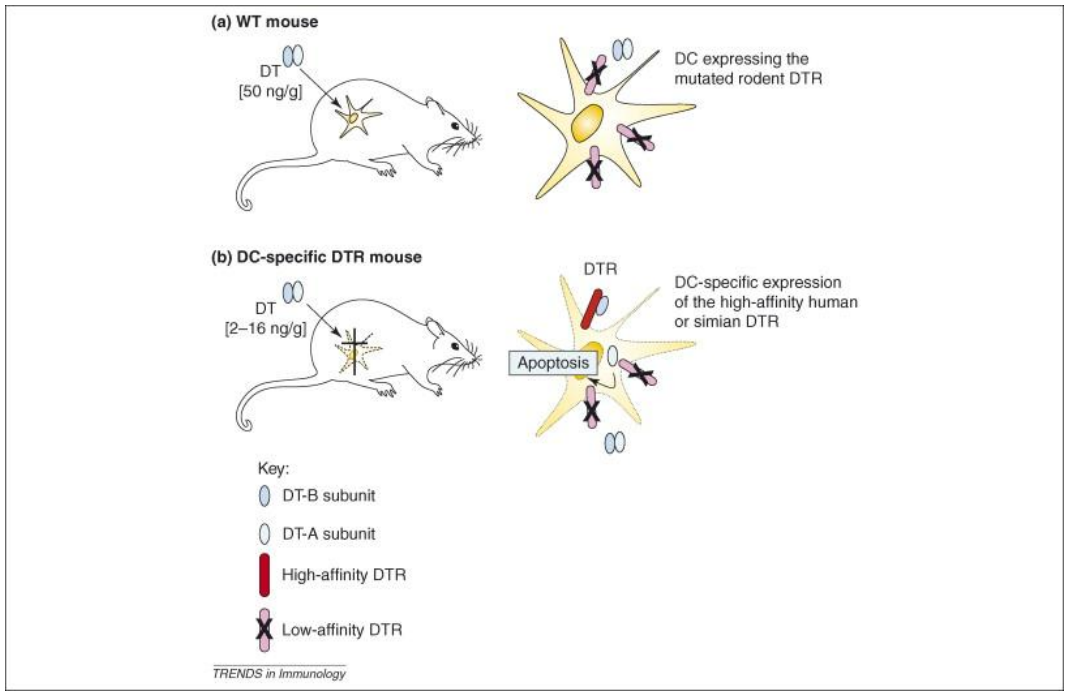

Figure 6: Depletion of CD11c positive cells in CD11c-DTR mice. Figure taken from Bennett *et al.*, 2007 [107].

4.3 *S. aureus* infection model

Mice were inoculated with 4 x 10^7 CFU of *S. aureus* in 150 µl of PBS via a lateral tail vein. For determination of bacterial loads in organs, infected mice were killed by CO_2 asphyxiation at different times of infection and the amount of bacteria was determined by preparing organ homogenates in 5 ml of PBS and plating 6-fold serial dilutions on blood agar (Invitrogen). Colonies were counted after incubation for 24 h at 37°C. For collection of serum, blood was collected at time of sacrifice, centrifuged at 4000 rpm for 10 min, and frozen at -80°C until use for further analysis.

4.4 Assessment of pathology

Lungs, kidneys, livers, and hearts were isolated from *S. aureus*-infected DCs-depleted and non-depleted CD11c-DTR mice at 24 h after bacterial inoculation, fixed in 10% neutral formalin, processed and embedded in paraffin. Four- to five-micrometer sections were prepared and stained with hematoxylin and eosin (H&E). The slides were examined by a pathologist who was blinded to the study groups. Histopathological lesions were graded for severity on a scale of 0 to 3. The scores were as follows: 0, non-pathological signs; 1, Multifocal PMNic interstitial pneumonia, focal purulent necrotizing myocarditis, or focal nephritis; 2, focal purulent abscess myocarditis, multifocal necrotizing hepatitis, focal purulent epicarditis, focal PMNic myocarditis; and 3, multifocal purulent necrotizing hepatitits.

4.5 Isolation of DCs from lungs and spleen

Spleens were meshed through a cell strainer and transformed in a single cell suspension. Lungs were minced with a scalpel and enzymatically digested with collagenase F and DNAse. Cell suspensions were incubated at 37°C for 30 min and reactions were stopped with 10 mM EDTA solution and red blood cells lysed after treatment with ammonium chloride. The percentage of CD11c/$CD11b^+$ DCs was assessed by flow cytometry. The absolute numbers of DCs was calculated by multiplying the total cell

counts by the percentage of CD11c/CD11b$^+$ cells. In some experiments, DCs were purified using anti-CD11c magnetic beads and positive selection MS columns (Miltenyi Biotec, Bergisch Gladbach, Germany). For this purpose, cells were resuspended in 400 µl of RPMI without supplements and 100 µl of CD11c micro beads (Miltenyi Biotec) were added to the cell suspension. After gently mixing and incubation for 15 min at 4°C, 2 ml of media was added and cells were centrifuged for 10 min at 1200 rpm. During this time, MS columns were placed in the magnetic field of a MACS separator and rinsed with 500 µl of medium. After centrifugation, supernatant was thoroughly removed and cells resuspended in 500 µl of medium. The cell suspension was applied to the MS columns and the flow-through discarded. Columns were washed three times with medium and flushed with 500 µl media to collect the retained cell population. The purity of the positive fraction was generally >90% as confirmed by CD11c staining and flow cytometry analysis. Purified splenic DCs were *in vitro* stimulated with 10^7 heat-killed *S. aureus* organisms or with 1 µM CpG for 16 h. The supernatant was used to determine the concentrations of cytokines.

4.6 Generation of bone marrow-derived DCs

Bone marrow cells were flushed from murine femurs and tibias. Progenitor cells (10^6/ml) were resuspended in RPMI 1640 containing 5% FCS, 10 ng/ml of recombinant mouse GM-CSF (granulocyte-macrophage colony-stimulating factor), and 2 ng/ml IL-4 (R&D Systems, Minneapolis, USA) and cultured for 6 days at 37°C in 5% CO_2. On days 2 and 4, DCs were gently washed and fed with fresh medium and supplementary factors. On day 6, the DC fraction was enriched using an OptiPrepTM (Axis-Shield, Oslo, Norway) gradient. Purified DCs were collected from the gradient interface and used for infection experiments. The purity of the resulting cell population consisted of >80% of DCs as determined by flow cytometry analysis using anti-mouse CD11c antibody (Becton Dickinson, San Jose, California, USA) and a FACSCaliburTM flow cytometer (Becton Dickinson).

4.7 Adoptive Transfer of DCs

Bone marrow-derived DCs from BALB/c mice were differentiated as described above and 10^6 immature or LPS-matured DCs were intravenously (i.v.) inoculated into *S. aureus*-infected BALB/c or DC-depleted CD11c-DTR mice at 16 h of infection. For DCs tracking experiments, DCs were labeled with 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) before adoptive transfer. For this purpose, up to $2x10^7$ DCs were incubated with 1 µM CSFE (Sigma) in 1ml PBS for 15 min at room temperature in the dark. The reaction was stopped by adding an equal amount of FCS. Labeled DCs were collected by centrifugation, washed twice with PBS and used for adoptive transfer experiments.

4.8 Collection of primary polymorph nuclear neutrophils

BALB/c mice were injected intraperitoneally with 1 mg of carrageenan 4 and 2 days prior experiment to deplete macrophages and to enrich the primary polymorph nuclear neutrophils (PMNs). Mice were euthanized and the abdominal cavity was flushed three times with 5 ml RPMI 1640 to collect the PMNs. Cells were centrifuged and directly used for experiments.

4.9 Partial depletion of PMNs *in vivo*

To induce a partial depletion of PMNs *in vivo*, mice were injected intravenously with 20 µg of anti-RB6 antibodies 24 h prior infection. This treatment results in the depletion of about 30% of PMNs as determined by flow cytometry.

4.10 Cytokine determination

Cytokine levels were determined in serum or lung homogenate by ELISA (BD Pharmingen, San Diego, CA, USA), using matched antibody pairs and recombinant cytokines as standards. 96-well flat-bottom microtitre plates were coated overnight at 4°C with 100 µl/well of the corresponding primary antibody diluted in coating buffer (0.05 M carbonate buffer, pH 9.6). The

coated plates were washed six times with washing solution (0.1% Tween 20/PBS) using an ELISA washer. After the protein solution was removed, plates were blocked with 200 µl per well of 10% FCS in PBS for 1 h at 37°C. The blocking solution was aspirated and serially diluted (1:2) serum samples or undiluted lung homogenates were added (100 µl/well) and incubated overnight at 4°C. After six washes with 0.1% Tween 20/PBS, secondary biotinylated antibodies were added and plates were further incubated for 2 h at 37°C. After several washing steps, 100 µl of HRP-conjugated streptavidin diluted 1:1000 in PBS supplemented with 10% FCS was added and plates were incubated for 30 min at room temperature. Plates were then washed 6 times and reactions were developed using 100 µl/well of Tetramethylbenzidine (TMB) solution. After appropriate incubation times, the reaction was stopped with 50 µl/ well of 2N H_2SO_4 solution and the absorbance at 450 nm with wavelength correction at 570 nm was determined using a Tecan ELISA Reader.

4.11 Flow cytometry

Cells were incubated with purified rat anti–mouse CD16/CD32 antibodies (Pharmingen) for 5 min to block Fc receptors and then stained with fluorescent antibodies against CD11c, CD11b, Gr-1, CD40, CD80, CD86, or MHC Class II for 30 min at 4°C. Labeled cells were analyzed by flow cytometry in a FACSCaliburTM (Becton Dickinson).

4.12 *S. aureus* infection of DCs

Bone marrow-derived DCs were plated in 48-well plates at a density of 5 x 10^6/ml and infected with *S. aureus* at a multiplicity of infection (MOI) of 2 bacteria per DC. Infected DCs were incubated for 2 h in antibiotic-free medium, washed twice with PBS to remove unbound bacteria and further incubated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS and 100 µg/ml of Gentamicin. DCs were collected by centrifugation at the indicated times after infection, washed with PBS and stained with antibodies for flow cytometry analysis.

4.13 Live cell imaging

For live-cell imaging, 10^6 DCs were seeded on 35-mm glass-bottom dishes (MatTek Corporation, USA) in DMEM 10% FCS, washed with PBS and subsequently maintained in imaging medium (DMEM without phenol red, 10% FCS, pH 7.4, 2 mM L-Glutamine). DCs were infected with GFP-expressing *S. aureus* at a MOI of 1 bacterium per DC. In some experiments, a solution of Lysotracker (Invitrogen, Germany) 1:20000 in DMEM imaging medium was added to label the DC acidic compartments. Imaging was started after 10 min of incubation using a Leica SP5 confocal microscope (Leica Microsystems, Germany) with AOBS and AOTF and equipped with environment control chamber (EMBLEM, Germany). During live imaging, single focal plane was monitored in time (xyt scanning mode) using 63x/1.4 HCX-PLAPO oil objective, Argon laser (488 nm) and DPSS laser (561 nm), scanner frequency 400 Hz; line averaging 4.

4.14 Image analysis

Processing of the stacks and analysis of the fluorescence intensity was performed using the ImageJ 1.38r software (Wayne Rasband, NIH USA). All time-lapse series were acquired in xyt scanning mode at a rate of 1 frame every 20 seconds.

4.15 Intracellular bacterial viability assay

To determine the amount of viable *S. aureus* within PMNs, Gr-1^+ cells were purified from the lungs of infected mice using magnetic beads (Miltenyi Biotec), treated with lysostaphin (5 µg/ml) for 15 min to eliminate extracellular bacteria and extensively washed to eliminate antibiotic traces. PMNs were collected by centrifugation, the cell pellet disrupted after treatment with dH_2O, and the amount of viable intracellular bacteria determined after serial plating onto blood agar.

4.16 Antigen-presentation assay

DCs were isolated from OT-II mice and differentiated as described in section 4.6. After purification, DCs (10^5/well) were infected with *S. aureus* or left uninfected. Some cells were furthermore co-incubated with 1mg/ml ovalbumin peptide. After 1 h, cells were treated with 5 µg/ml Lysostaphin for 15 min to eliminate non-phagocytosed bacteria. After washing, cells were resuspended in fresh media containing 100 µg/ml Gentamicin and further incubated for 6 h. DC were then harvested and seeded into a 96-well cell culture plate (10^5/well). Purified $CD4^+$ T cells isolated from spleen and lymph nodes of OT-II mice were added (5 x 10^5/well) and the cultures were incubated for 3 days at 37°C, 5% CO_2. For isolation and purification of $CD4^+$ T cells, spleen and lymph nodes were transformed in a single cell suspension and incubated with anti-CD4-PE antibodies (dilution 1:100) for 15 min at 4°C. After washing, anti-PE-magnetic beads and labeled cells were isolated following the procedure described in section 4.5. After 3 days of culture, 50 µl of fresh media supplemented with 1 µC [methyl-^{3}H]Thymidine was added and cells were incubated for further 18 h. Cells were harvested on glass fibre filters by using the Inotech cell harvester. Results were expressed as the arithmetic mean of [methyl-^{3}H]Thymidine uptake in counts per minute (cpm).

4.17 Statistical analysis

Data were analyzed using GraphPad Prism 4.0 (GraphPad software). Unless otherwise specified, all data are presented as mean standard deviation (± SD). Comparison between groups was made by use of two-tailed *t*-test. Comparison of survival time curves was performed by use of Logrank test. P values <0.05 were considered as significant.

5. Results

5.1 Phagocytic uptake of *S. aureus* by DCs

DCs are generally present in all peripheral tissues and accumulate at the sites of pathogen entry where they can efficiently phagocyte the invading pathogens. DCs could be one of the first cell populations interacting with *S. aureus* after bacterial invasion. The capacity of bone marrow-derived murine DCs to uptake *S. aureus* was investigated by immunofluorescence live-cell imaging. DCs were stained red with lysotracker, which stains acidic cellular compartments, and then incubated with GFP-expressing *S. aureus* at a MOI of 1:1. Stills of a representative movie (Video S1, see appendix I. Videos) are shown in Figure 7. DCs began to internalize *S. aureus* 30 min after co-incubation (Figure 7A, white arrow) and bacterial phagocytosis continued unabated (Figure 7B-D, white arrow).

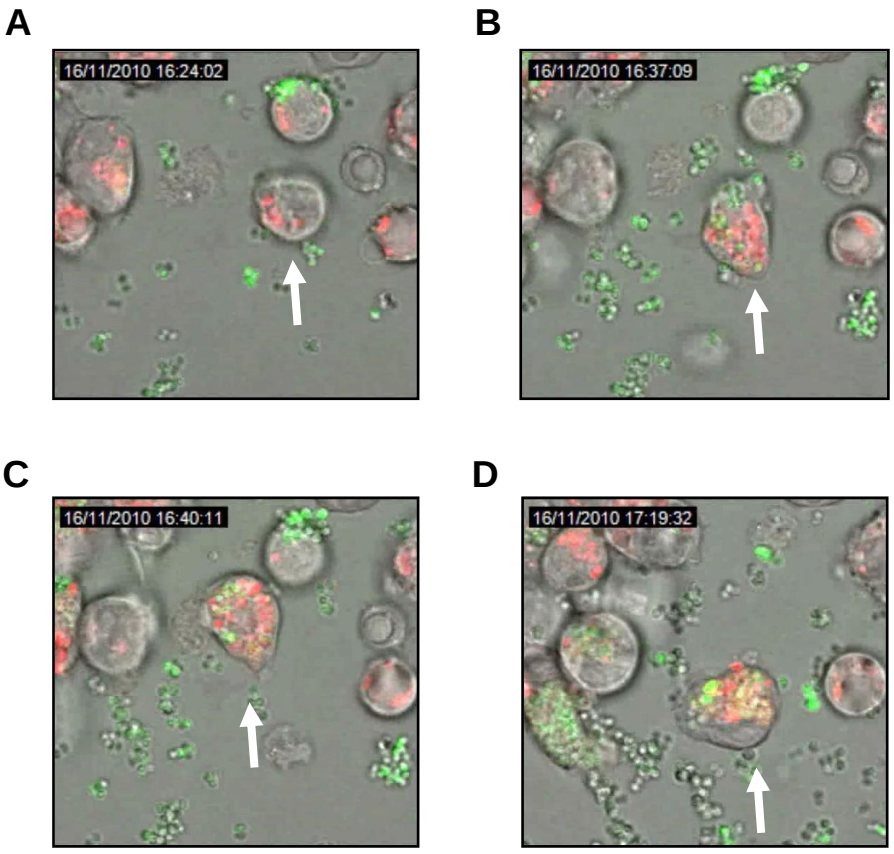

Figure 7: Phagocytic uptake of *S. aureus* by DCs. Stills from a representative movie (Video S1) showing phagocytosis of *S. aureus* by a DC (indicated by the white arrow). Bacteria are labeled green (GFP) and the DCs are labeled red with lysotracker.

After 1 h of infection, a large proportion of the internalized bacteria appear in yellow indicating co-localization within acidic phagocytic vacuoles. The red and green fluorescence associated with DCs were then quantified using ImageJ software analysis, which plot the mean fluorescence of representative cells in consecutive frames obtained from a live-cell imaging video. Representative frames at time 0 and 64 minutes (min) of live imaging are displayed in Figure 8A. As shown in Figure 8B, fluorescence associated with *S. aureus* significantly increased in sequential frames confirming the continuous bacterial uptake by the DCs. Furthermore, the intensity of red fluorescence (lysotracker) also increased in a chronological manner indicating a progressive acidification of the phagosomes within the DCs (Figure 8C).

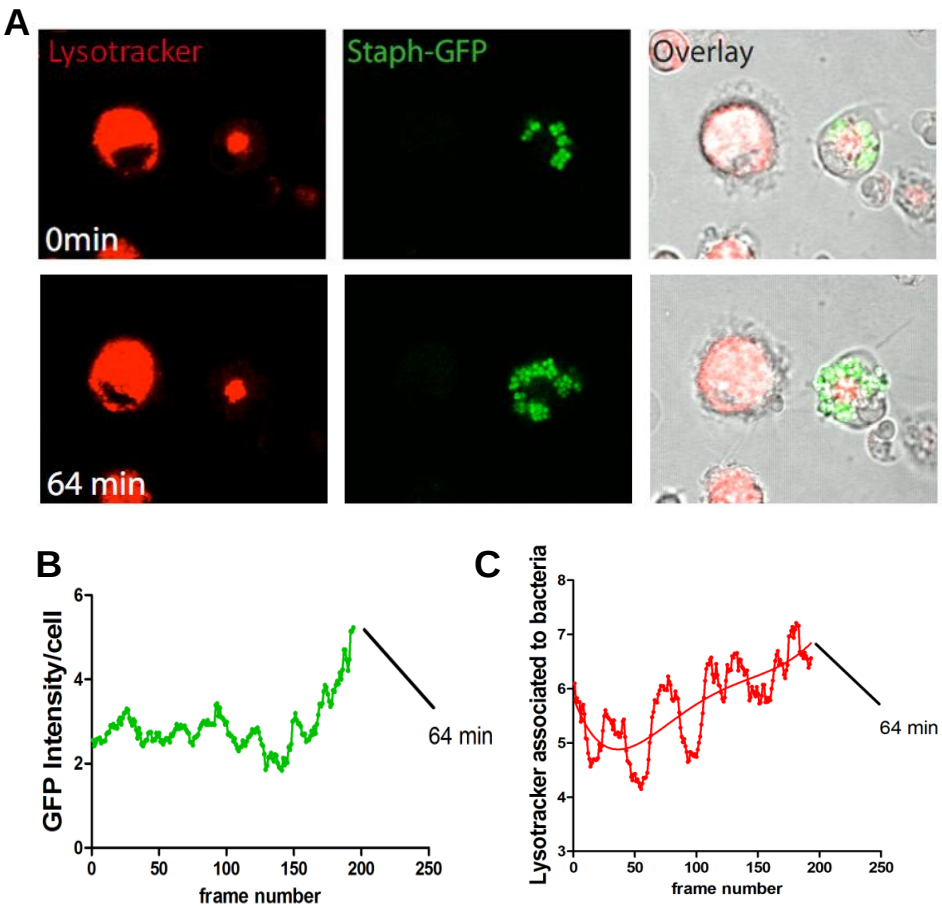

Figure 8: Phagosome acidification in *S. aureus*-infected DCs. (A) Representative frames at time 0 (upper panels) and 64 (lower panels) min of a live-cell video showing lysotracker (red), *S. aureus* (green) and the overlaid image. Fluorescence profile of GFP (B) and lysotracker (C) were analyzed frame by frame using ImageJ software.

5.2 *S. aureus* induces maturation of DCs

Internalization of pathogens can subsequently trigger DC maturation and migration from peripheral tissues to lymphoid organs. The process of DC maturation, in general, involves a redistribution of MHC Class II from intracellular endocytic compartments to the DC surface, an increase in the surface expression of co-stimulatory molecules as well as secretion of cytokines. Therefore, the ability of *S. aureus* to induce maturation of DCs was determined by measuring the up-regulation of the surface maturation markers CD40, CD80, CD86, and MHC class II at 6 h after bacterial infection. To this end, murine bone marrow-derived DCs were incubated with *S. aureus* for 2 h, extracellular bacteria killed by addition of Gentamicin, and DCs were further incubated for 6 h. Uninfected DCs were used as negative control and DCs stimulated with 2 µg/ml of LPS were used as positive control. After 6 h of culture, DCs were labeled with FITC-conjugated monoclonal antibodies to CD40, CD80, CD86, or MHC class II molecules and PE-conjugated antibodies to CD11c, and analyzed by flow cytometry. The $CD11c^+$ cell population was gated and analyzed for the expression of CD40, CD80, CD86, and MHC class II expression. A significant increase in the mean fluorescence intensity (MFI) of the different maturation markers was detected in *S. aureus*-infected DCs when compared with DCs cultured with medium alone (CTR) (Figure 9A). The average level of maturation markers induced by *S. aureus* was significantly greater than that induced by LPS after 6 h of stimulation (Figure 9A). In addition, the capacity of *S. aureus* to induce IL-6, IL-12, and TNF-α secretion by DCs was also evaluated. For this purpose, the levels of these cytokines were determined in the culture supernatant of *S. aureus*-infected DCs by ELISA. *S. aureus* was found to induce the release of high levels of all these pro-inflammatory cytokines with the mean values comparable to those induced by LPS (Figure 9B).

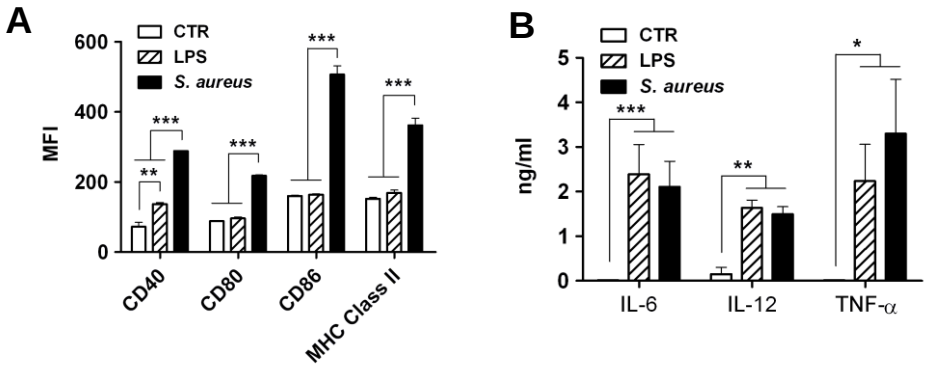

Figure 9: *S. aureus* induces maturation of DCs. (A) Mean fluorescence intensity (MFI) of CD40, CD80, CD86, or MHC class II molecules on DCs left untreated (white bars), stimulated with LPS (hatched bars) or infected with *S. aureus* (black bars) determined by flow cytometry analysis. (B) Levels of IL-6, IL-12 and TNF-α determined in the culture supernatant by ELISA. Each bar represents the mean ± SD of triplicates from three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

5.3 The mouse model of *S. aureus* infection

A murine model of *S. aureus* bacteremia was then used to determine the relevance of DCs for host response against *S. aureus*. In this infection model, BALB/c mice were intravenously inoculated with 4 x 10^7 CFU of *S. aureus* and the course of infection was monitored by determination of weight loss, survival, and bacterial burdens in systemic organs at progressive times after bacterial inoculation. Shortly after infection, all mice displayed overt clinical signs of morbidity as determined by a decrease in their physical activity, a tendency to huddle, piloerection, and gradual weight loss (Figure 10A). Infected mice showed a progressive mortality starting at day 2 post-inoculation, with 100% mortality by day 4 of infection (Figure 10B) and progressive bacterial growth in kidneys, liver and lungs (Figure 10C). In addition, serum levels of IL-6, an early marker of gram-positive sepsis [108], increased progressively in *S. aureus*-infected mice (Figure 10D). Therefore, the inability of BALB/c mice to clear *S. aureus* infection results in an overwhelming systemic inflammatory response that culminates in the development of sepsis and death.

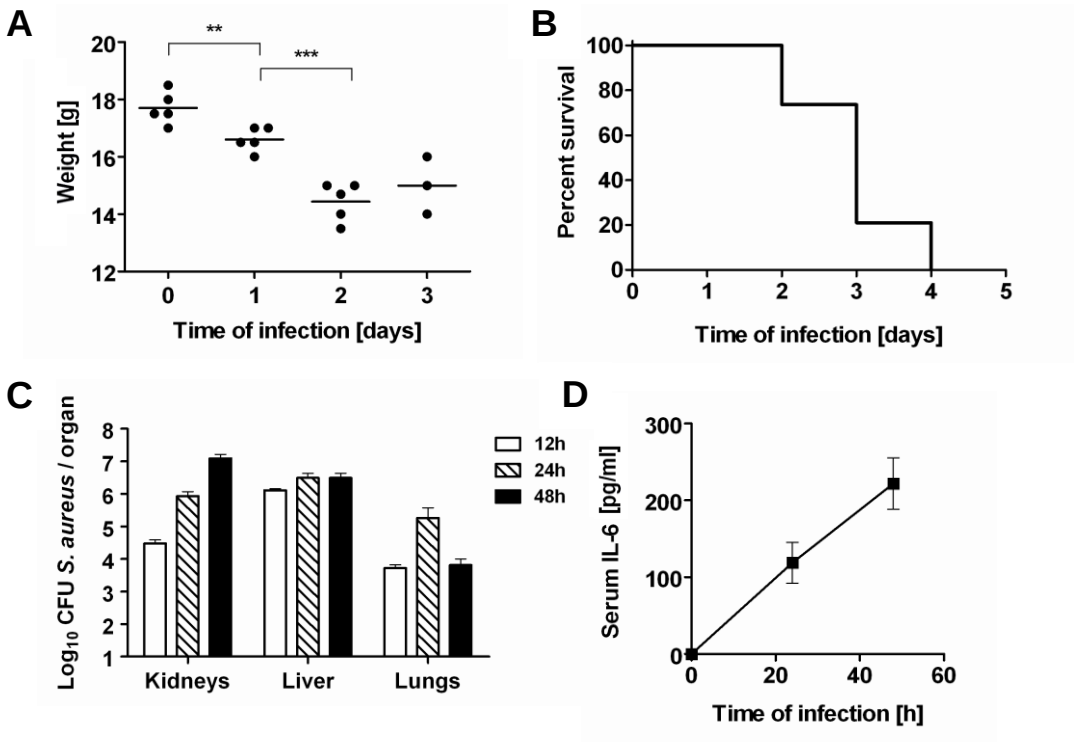

Figure 10: The course of *S. aureus* infection in BALB/c mice. Body weight monitoring (A), survival curves (B), bacterial loads in systemic organs (C) and serum levels of IL-6 (D) in BALB/c mice after intravenous infection with 4 x 10^7 CFU of *S. aureus*. **, $p < 0.01$; ***, $p < 0.001$.

5.4 DCs are recruited into the organs of *S. aureus*-infected mice

As depletion of DCs in secondary lymphoid organs has been reported at the early phase of polymicrobial sepsis in mice [109, 110], the number of DCs in the spleen of *S. aureus*-infected mice was determined at 24h and 48 h post bacterial inoculation by flow cytometry analysis. In contrast to the reduction of DCs observed in the spleen of mice during polymicrobial sepsis, the number of DCs ($CD11c^{high}/CD11b^{high}$) increased significantly in the spleen of *S. aureus* septic animals (Figure 11A). Recruitment of DCs also took place in peripheral organs such as in the lungs (Figure 11B).

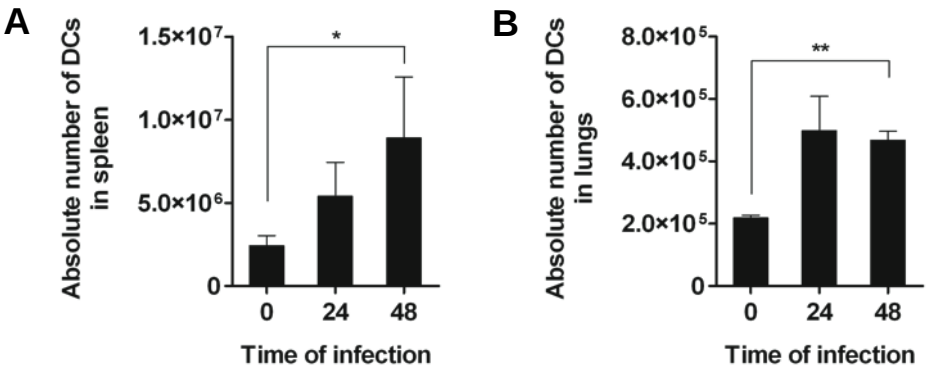

Figure 11: DCs are recruited into the spleen and lungs of *S. aureus*-infected mice. BALB/c mice were infected with 4 x 10^7 CFU of *S. aureus* and the amount of DCs determined in the spleen (A) and lungs (B) at 24 h and 48 h of infection. Each bar represents the mean ± SD of 5 mice per time point. *, $p < 0.05$; **, $p < 0.01$.

The functional capacity of the recruited DCs was then determined by isolating DCs from the organs of infected mice and re-stimulating them *in vitro* for 16 h with 10^7 cells of heat-killed *S. aureus.* The production of IL-12, TNF-α, and IL-6 was measured in the culture supernatant of the re-stimulated DCs by ELISA. As shown in Figure 12, the DCs isolated from infected mice produced significantly higher levels of IL-12 (Figure 12A), TNF-α (Figure 12B) and IL-6 (Figure 12C) than those isolated from uninfected animals. Similar results were obtained after re-stimulating the isolated DCs with the TLR-9 ligand CpG (data not shown). These results indicate that DCs did not lose their capacity to produce cytokines in *S. aureus*-infected mice and that they were in a more advanced state of maturation than DCs isolated from uninfected animals.

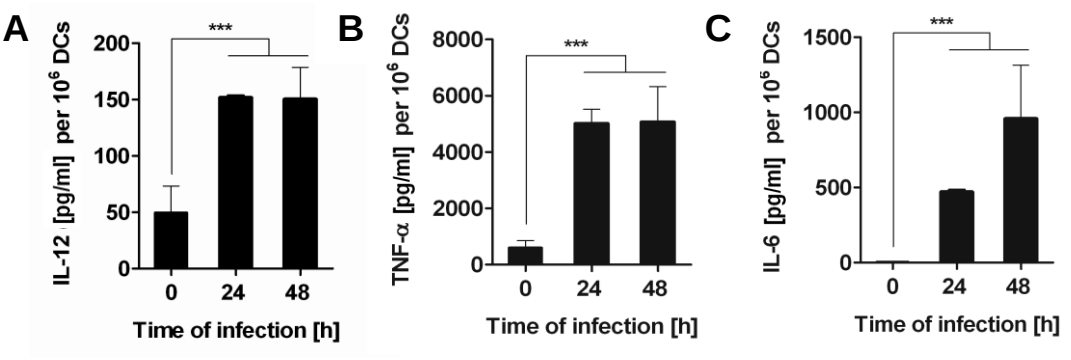

Figure 12: Cytokines released by DCs isolated from the organs of infected mice after *in vitro* re-stimulation with heat-killed *S. aureus*. DCs were purified from the spleen of *S. aureus*-infected mice at 24 h after bacterial inoculation and stimulated with 10^7 heat-killed *S. aureus* for 16 h. Release of IL-12 (A), TNF-α (B) and IL-6 (C) in the culture supernatant were quantified by ELISA. Each bar represents the mean ± SD of triplicates from three independent experiments. ***, $p < 0.001$.

5.5 Depletion of DCs exacerbates the severity of *S. aureus* infection

To determine the relevance of the recruited DCs for host defense against *S. aureus* infection, CD11c-DTR mice were used to selectively deplete DCs. As described in the Materials and Methods section, these transgenic mice express the diphtheria toxin (DT) receptor on the CD11c promoter region, which allows for selective depletion of DCs following the administration of DT. It was observed that CD11c-DTR mice depleted of DCs exhibited significant reduced survival time (Figure 13A) and greater numbers of bacteria in the organs (Figure 13B) than non-depleted animals. In correlation with the accelerated mortality, DC-depleted mice exhibited higher pathology scores than non-depleted animals (Figure 13C). To exclude a potential effect of the DT treatment *per se* on the course of infection, BALB/c mice were treated with DT accordingly to the protocol used for the CD11c-DTR mice and the bacterial burden in systemic organs was determined. No differences were observed in the amount of bacteria between DT-treated and untreated BALB/c mice (Figure 13D).

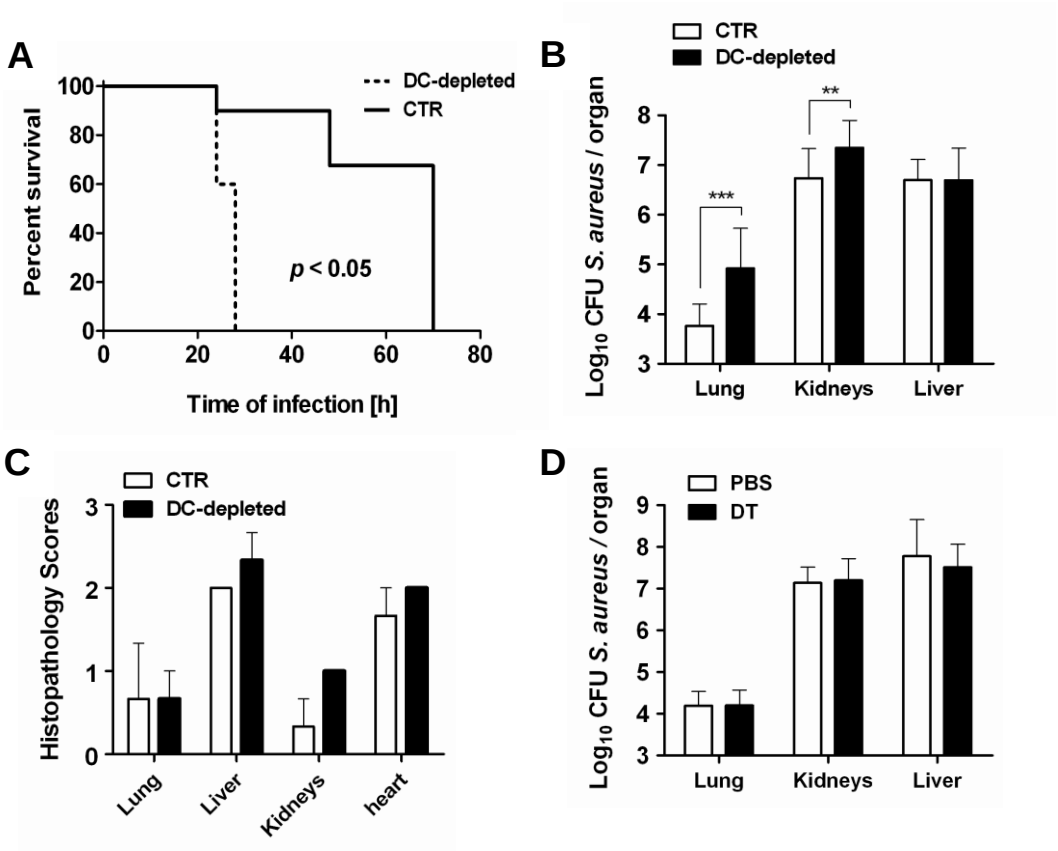

Figure 13: Depletion of DCs aggravates the severity of *S. aureus* infection. (A) Survival curves of DCs-depleted (broken line) and non-depleted (continuous line) mice after intravenous inoculation with *S. aureus*. Bacterial loads (B) and pathology scores (C) in the organs of DCs-depleted (black bars) and non-depleted (white bars) mice at 24 h after bacterial inoculation. (D) Bacterial loads in the organs of DT-treated (black bars) or PBS-treated (white bars) BALB/c mice at 24 h after *S. aureus* inoculation. Data are representative of three independent experiments. **, $p < 0.01$; ***, $p < 0.001$.

5.6 Reconstitution of DCs-depleted mice by adoptive transfer of DCs reverses the effect of DC depletion in the course of *S. aureus* infection

Van Rijt *et al.* reported that treatment of CD11c-DTR mice with DT also induces the depletion of alveolar macrophages since this cell population expresses high levels of CD11c [111]. Therefore, the effect of DT treatment in the alveolar macrophage population of CD11c-DTR mice was investigated. CD11c and CD11b markers were used to differentiate alveolar macrophages ($CD11c^{high}/CD11b^{low}$) from lung DCs ($CD11c^{intermediate}/CD11b^{high}$). The results show that treatment with DT in-

duced extensive depletion of alveolar macrophages (Figure 14B, Gate 1) in addition to the depletion of lung DCs (Figure 14B, Gate 2),

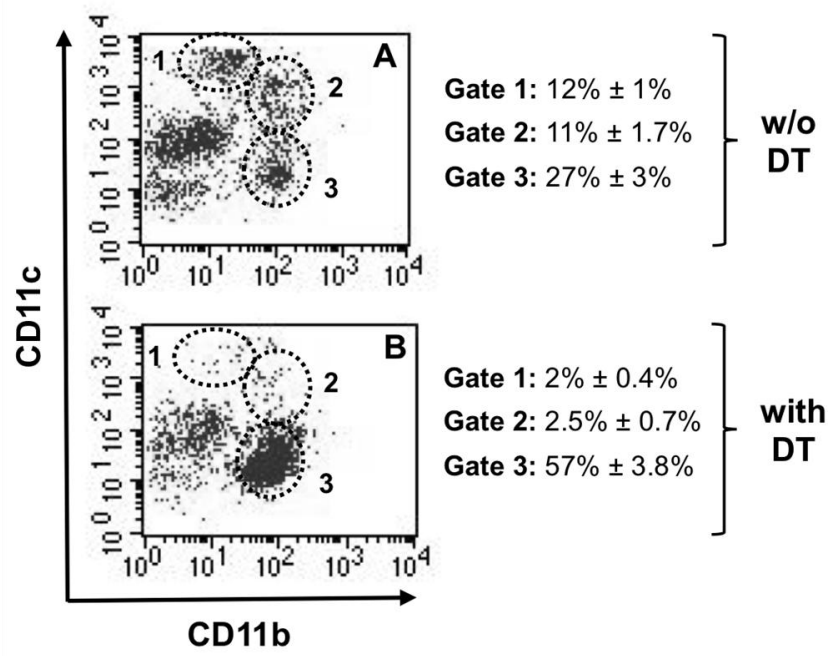

Figure 14: Depletion of alveolar macrophages in CD11c-DTR mice after treatment with DT. Flow cytometry analysis of lung cells from CD11c-DTR transgenic mice treated with either PBS (A) or with DT (B) and infected intravenously with *S. aureus* 24 h thereafter. Lung cell populations were analyzed 24 h after bacterial inoculation. A single-cell suspension was obtained after enzymatic digestion of lung tissue and alveolar macrophages (Gate 1), DCs (Gate 2) and PMNs (Gate 3) identified by their relative expression of CD11c and CD11b.

To determine whether the loss of DCs, but not of alveolar macrophages was directly responsible for the exacerbation of infection, CD11c-DTR mice were adoptively transferred with DCs obtained from normal BALB/c mice. The capacity of adoptively transferred matured or immature DCs to migrate into the spleen (Figure 15A) and lungs (Figure 15B) was confirmed by flow cytometry analysis using CFSE-labeled DCs. As shown in Figure 16, reconstitution with DCs returned the capacity of CD11c-DTR mice to control the infection in the lungs and kidneys.

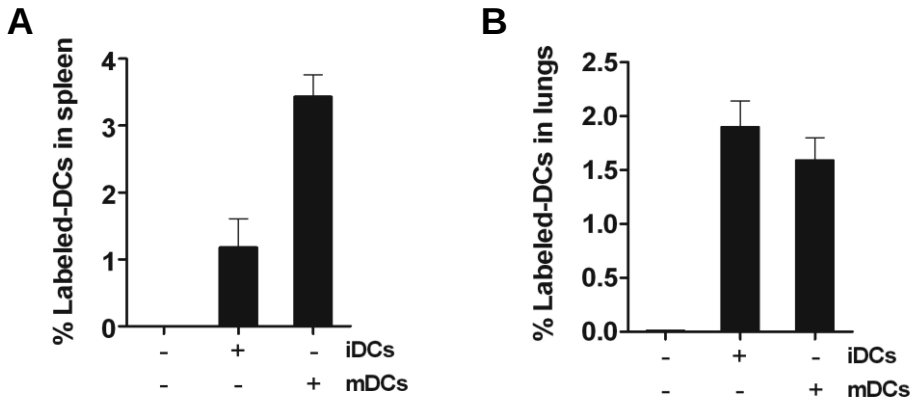

Figure 15: Tracing of adoptively transferred CFSE-labeled DCs by flow cytometry. *S. aureus*-infected mice were intravenously injected with either mature or immature CFSE-labeled DCs and single cells released from whole spleen (A) or lung (B) digestion were analyzed by flow cytometry. Each bar represents the mean ± SD percentage of CFSE-stained DCs.

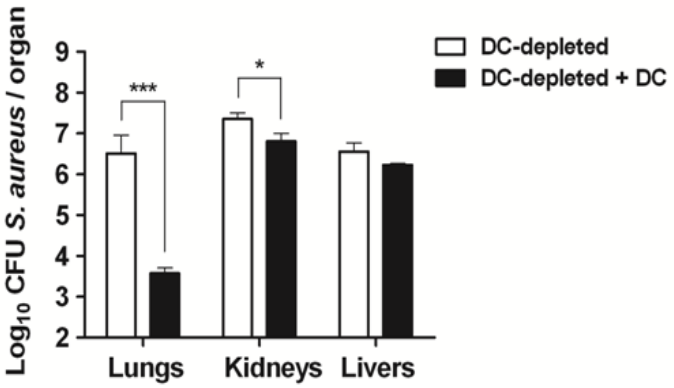

Figure 16: DC reconstitution improves the capacity of DCs-depleted CD11c-DTR mice to control *S. aureus* infection. Bacterial loads in the lungs, kidneys and livers of CD11c-DTR mice treated with DT 24 h prior inoculation with *S. aureus* and intravenously injected at 16 h of infection with 10^6 DCs obtained from BALB/c mice. CFU were determined in the different organs at 24 h after DCs transfer. Each bar represents the mean ± SD of three independent experiments. *, $p < 0.05$; ***, $p < 0.001$.

5.7 Adoptive transfer of DCs improves the course of infection in BALB/c mice

As depletion of DCs resulted in disease exacerbation, it can be hypothesized that increasing the amount of DCs could improve the course of infection in BALB/c mice and thus be used as a therapeutic intervention. To test this assumption, BALB/c mice were injected with 10^6 immature or LPS-matured DCs 16 h after bacterial inoculation and the bacterial loads were determined in the systemic organs 24 h after the injection of DCs. Results in Figure 17 show that both immature and mature DCs improved the capacity of BALB/c mice to control *S. aureus* in the lungs.

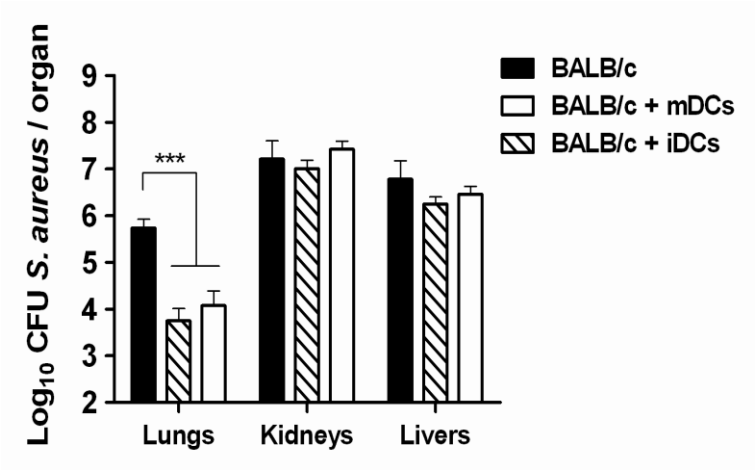

Figure 17: Effect of adoptive transfer of DCs on the capacity of BALB/c mice to control *S. aureus* infection. Bacterial loads in the lungs, kidneys, and livers of BALB/c mice intravenously injected at 16 h after bacterial inoculation with either 10^6 mature DCs (white bars), 10^6 immature DCs (hatched bars), or PBS (black bars). CFU were determined in the different organs at 24 h after DCs transfer. Each bar represents the mean ± SD of three independent experiments. ***, $p < 0.001$.

5.8 DCs do not contribute to direct killing of *S. aureus*

The next step was to investigate the molecular mechanisms underlying the beneficial effect afforded by DCs during experimental *S. aureus* infection. As DCs are phagocytic cells, the ability of DCs to contribute to direct killing of *S. aureus* was evaluated. For this purpose, DCs were infected with *S. aureus* at a MOI of 1:10 and the amount of intracellular bacteria determined at progressive times of infection. The results in Figure 18 show that *S. aureus* survived within the DCs.

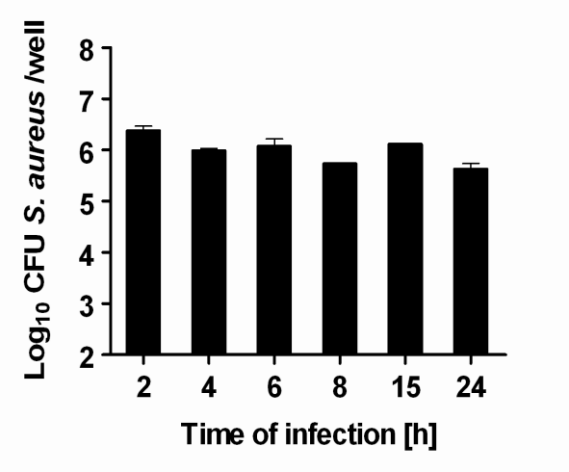

Figure 18: *S. aureus* survives within DCs. DCs were infected with *S. aureus* and the number of viable intracellular bacteria was determined at progressive times of infection by lysing host cells, plating the lysates on agar plates, and counting the number of colonies.

Live-cell imaging analysis of infected DCs demonstrated that *S. aureus* was not only capable to survive but it was also capable to replicate within the DCs (Figure 19).

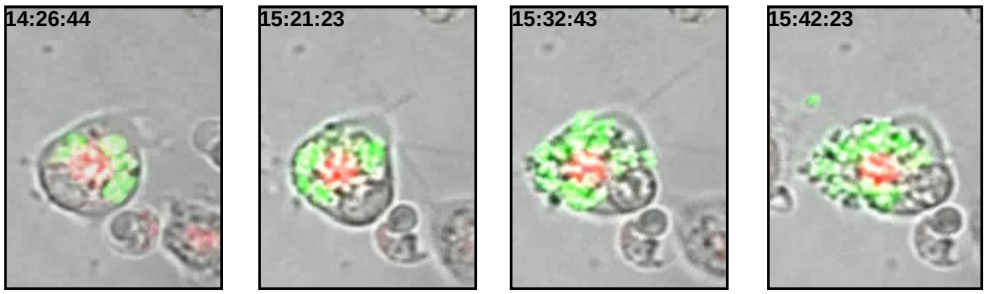

Figure 19: *S. aureus* replicates within the DC. Examples of real time movie (Video S2, see appendix I**. Videos**) images of a DC infected with GFP-expressing *S. aureus* in a photographic sequence lasting for approximately 1 h and 15 min.

5.9 Depletion of DCs leads to an enhanced recruitment of PMNs into the lungs of infected mice

As a direct contribution of DCs to bacterial killing was excluded, the possibility that the beneficial effect of DCs was due to their involvement in the recruitment of inflammatory cells involved in bacterial clearance was investigated. For this purpose the lungs were removed from DCs-depleted and non-depleted mice before and 24 h after *S. aureus* inoculation, digested and the amount of PMNs was determined by flow cytometry analysis. The results demonstrate an enhanced recruitment of PMNs into the lungs of DC-depleted animals, not only in percentage (Figure 14, Gate 3), but also in total numbers (Figure 20).

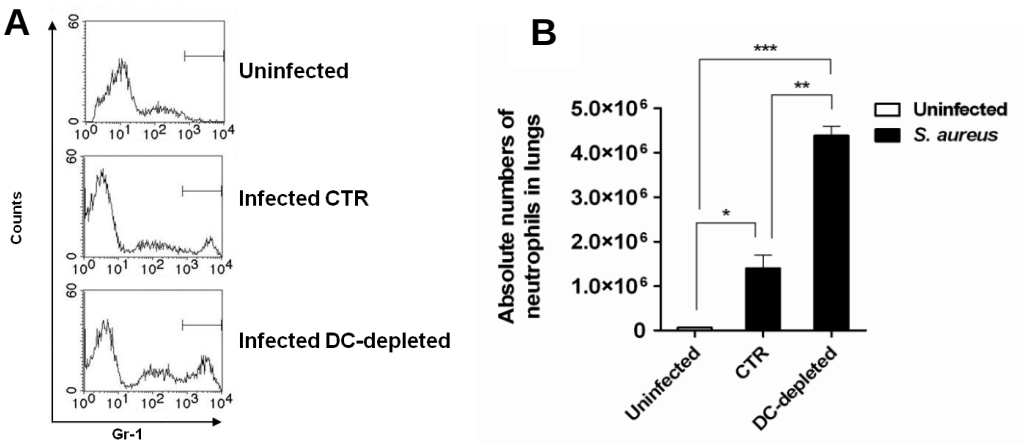

Figure 20: DC-depletion is associated with an increased recruitment of PMNs in the lungs of *S. aureus*-infected mice. (A) Representative histograms for expression of Gr-1 molecule (PMNs) in the lung cells of uninfected (upper panel), *S. aureus*-infected and PBS-treated (middle panel) or *S. aureus*-infected and DT-treated (lower panel) CD11c-DTR mice at 24 h after bacterial inoculation. (B) Absolute number of PMNs recruited into the lungs in the above-mentioned groups. Each bar represents the mean ± SD of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

5.10 PMNs recruited within the lungs of DC-depleted mice harbor higher numbers of viable intracellular bacteria than PMNs within the lungs of non-depleted mice

Since several publications have reported that PMNs contribute to the clearance of *S. aureus* infections [62-64], higher numbers of PMNs should correlate with better bacterial clearance rather than with increased bacterial loads. However, other studies have also reported that increased bacterial burdens at the site of *S. aureus* infection correlated with excessive numbers of migrating PMNs harboring intracellular viable bacteria [112, 113]. To determine if the PMNs infiltrating the lungs of *S. aureus*-infected mice harbored viable bacteria, PMNs were isolated out of the lungs of depleted and non-depleted CD11c-DTR mice, treated with lysostaphin to eliminate extracellular bacteria and plated. The results in Figure 21A show, that significantly higher number (20-fold) of viable *S. aureus* cells were found in the PMN population isolated from the lungs of DC-depleted than from the lungs of non-depleted mice. More interestingly was the observation that the amount of viable *S. aureus* per PMN was also significantly higher (10-fold) in the PMNs isolated from the lungs of DC-depleted than non-depleted mice (Figure 21B).

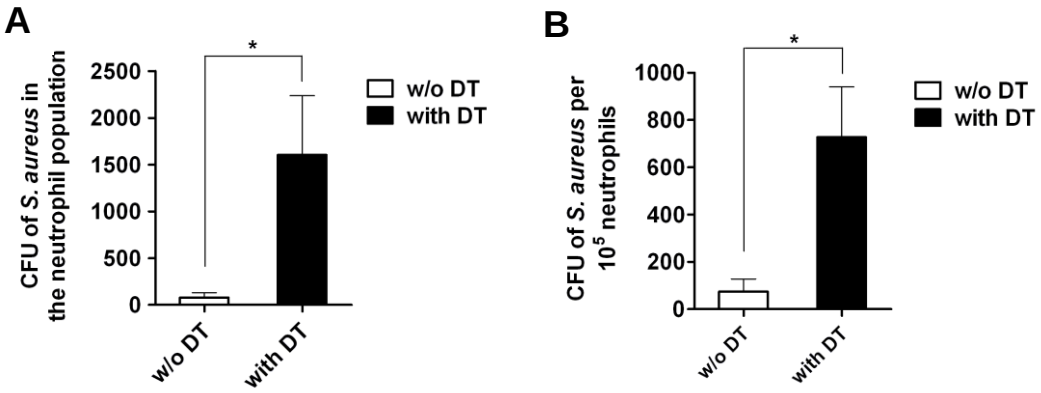

Figure 21: Higher amount of viable *S. aureus* are present within the PMNs recruited into the lungs of DC-depleted than in those of non-depleted mice. Quantification of viable intracellular *S. aureus* in the total population of PMNs (A) or per 10^5 PMNs (B) in the lungs of *S. aureus*-infected DT-treated (black bars) or PBS-treated (white bars) CD11c-DTR mice at 24 h after bacterial inoculation. Each bar represents the mean ± SD of three independent experiments. *, $p < 0.05$.

5.11 Higher levels of CXC chemokines are produced in the lungs of DC-depleted than in the lungs of non-depleted *S. aureus*-infected mice

Because CXC chemokines such as KC and MIP-2 have been reported not only to promote PMN migration but also the uptake of *S. aureus* into a compartment that permits its survival within PMNs [112], the levels of KC and MIP-2 in the lungs of *S. aureus*-infected mice in the presence and absence of DCs were determined. A significantly increased KC (Figure 22A) and MIP-2 (Figure 22B) concentration in the lungs of DC-depleted mice compared with non-depleted animals was observed.

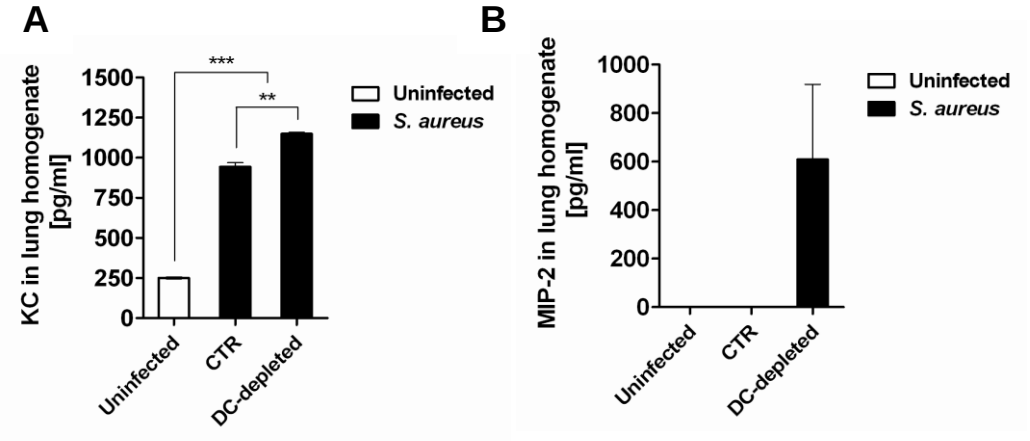

Figure 22: Amount of CXC chemokines in the lungs of *S. aureus*-infected mice in the presence or absence of DCs. Whole-lung KC (A) and MIP-2 (B) levels in uninfected (white bars) and *S. aureus*-infected (black bars) either DT-treated or PBS-treated CD11c-DTR mice at 24 h after bacterial inoculation. The levels of KC and MIP-2 were determined in lung homogenates by ELISA. Each bar represents the mean ± SD of three independent experiments. **, $p < 0.01$; ***, $p < 0.001$.

These findings suggest that DCs influence both the recruitment of PMNs into the tissue of *S. aureus*-infected mice as well as the antimicrobial capacity of these phagocytic cells via the regulation of lung KC and MIP-2 production.

5.12 Partial depletion of PMNs does not reverse the detrimental effect of DC depletion

Gresham *et al.* [112] reported that partial depletion of PMNs in *S. aureus*-infected mice resulted in lower amount of bacteria in organs and increased survival rate. To determine if partial depletion of PMNs can reduce the bacterial burdens in the experimental setting used in the study presented here, DCs-depleted mice were treated with 5 µg of anti-RB6 antibodies to achieve a partial depletion of circulating PMNs (30% of depletion) prior to intravenous infection with *S. aureus*. As shown in Figure 23, a partial depletion of PMNs did not influence the bacterial loads in the organs of DC-depleted mice determined at 24 h after bacterial inoculation.

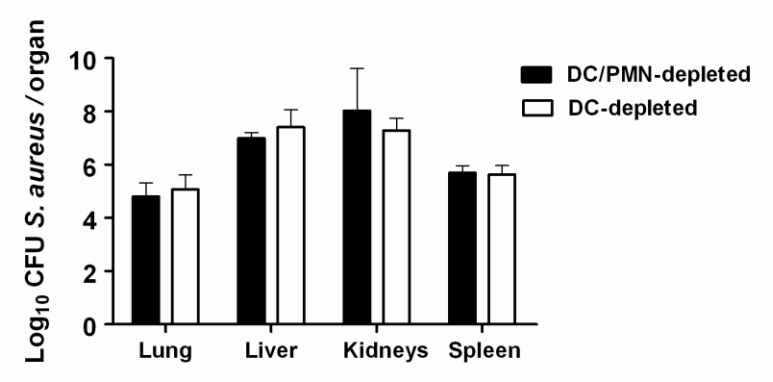

Figure 23: Effect of partial depletion of PMNs on bacterial burdens in the organs of DC-depleted mice. Bacterial loads in the organs of DCs-depleted mice, which have been partially depleted of PMNs (black bars) or left untreated (white bars) at 24 h after intravenous inoculation with *S. aureus.* Each bar represents the mean ± SD of two independent experiments.

5.13 Influence of *S. aureus* in the capacity of DCs to stimulate naïve antigen-specific $CD4^+$ T cells

DCs are potent antigen-presenting cells (APCs) that possess the ability to stimulate naïve T cells. To determine the influence of *S. aureus* in the capacity of DCs to stimulate naïve antigen-specific $CD4^+$ T cells, DCs (either infected or uninfected) were loaded with $OVA_{323-339}$ or left untreated and co-cultivated with $CD4^+$ T cells from OT-II mice, which are specific for this peptide. A significantly higher proliferation was observed when $CD4^+$ T

cells were co-cultivated with *S. aureus*-infected OVA-loaded DC in comparison to those co-cultivated with uninfected OVA-loaded DCs (Figure 24).

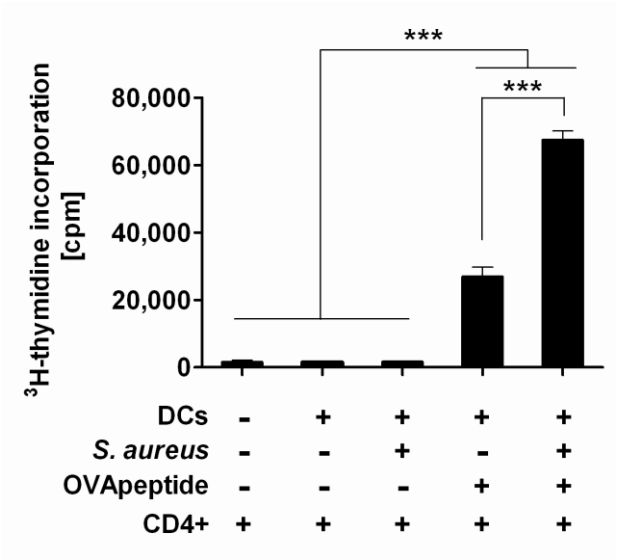

Figure 24: Proliferation of naïve CD4$^+$ T cells after co-cultivation with uninfected or *S. aureus*-infected OVA-loaded DCs. CD4$^+$ T cells from OT-II transgenic mice were co-cultivated with uninfected or *S. aureus*-infected, OVA-loaded or unloaded DCs. The proliferative capacity of CD4$^+$ T cells was measured by the incorporation of [methyl-^{3}H]Thymidine. Each bar represents the mean cpm ± SD of triplicates. Result from one single experiment is shown. ***, $p < 0.001$.

6. Discussion

During the last decades, an increasing number of infections caused by *S. aureus* has been reported worldwide [1, 2]. The increasing prevalence of staphylococcal infections is a direct consequence of the continuing evolution of antimicrobial resistance among *S. aureus* strains, added to the increased use of implantable devices and the boosting in the number of patients with immunocompromised status because of HIV infection or immunosuppression after transplantation or cancer treatment [114-116]. Therefore, new therapeutic options with novel models of actions that may circumvent antibiotic resistance are required to tackle the problem posed by *S. aureus* in a more effective way. In this regard, therapeutic approaches aimed to enhance the efficiency of the host immune response to eliminate *S. aureus* may represent the best option. However, a more precise knowledge of the immune mechanisms involved in host defense against *S. aureus* is required in order to understand how the immune system can be manipulated to achieve a more efficient control of infection.

In this work, the role of DCs, a subset of cells unique in their capacity to regulate the immune response, in the pathogenesis of *S. aureus* infections was investigated.

First, the interactions between DCs and *S. aureus* were evaluated using live-cell imaging microscopy (see section 5.1). The results of these experiments demonstrate the remarkable capacity of DCs to trace and phagocyte *S. aureus*. After encounter *S. aureus*, DCs initiated a maturation process as shown by the up-regulation of co-stimulatory molecules CD40, CD80, CD86, and MHC class II as well as the release of proinflammatory cytokines IL-6, IL-12, and TNFα.

A murine model of infection was used to investigate the behavior of DCs during *S. aureus* infection under *in vivo* conditions. In this model, BALB/c mice were intravenously infected with *S. aureus* strain SH1000 as previously described [64]. Examination of the amount of DCs in lungs and spleen of infected mice at progressing times after bacterial inoculation demonstrated that DCs were mobilized and actively recruited into infected

tissue during the course of infection. Isolation and *in vitro* re-stimulation of the recruited DCs with heat-killed *S. aureus* or with the TLR-9 ligand CpG revealed that these cells were in a more advance stage of activation than DCs from uninfected animals since they produced significantly higher levels of inflammatory cytokines.

The next step was to determine the relevance of DCs for host defense against *S. aureus* infection. For this purpose, CD11c-DTR transgenic mice, which allows for selective depletion of DCs [105], were used. Depletion of DCs resulted in substantial worsening of pathogen clearance, particularly in the lungs and kidneys, and in accelerated mortality of infected animals. In accordance with this data, a recent study reported that depletion of DCs in mice intranasally inoculated with *S. aureus* was associated with an increased bacterial load in the lungs [117]. To exclude an influence of the DT itself on the course of *S. aureus* infection, BALB/c mice were treated with DT before infection in a similar way as CD11c-DTR mice were treated and bacterial loads were monitored. Treatment with DT alone had no effect on the course of infection confirming that the observed exacerbation of infection was indeed caused by DC depletion and not by side effects of DT treatment.

As the lung was the most affected organ by DC depletion and it has been shown that alveolar macrophages can also be depleted by DT treatment [111], the potential depletion of alveolar macrophages by DT treatment in the experimental settings used in this study was evaluated. The CD11c and CD11b markers were used to differentiate alveolar macrophages ($CD11c^{high}/CD11b^{low}$) from lung DCs ($CD11c^{intermediate}/CD11b^{high}$) by FACS analysis. The results show that, in addition to the depletion of lung DCs, treatment with DT induced extensive depletion of alveolar macrophages (Figure 14). To determine whether the loss of DCs but not of alveolar macrophages was directly responsible for the exacerbation of infection, DC-depleted CD11c-DTR mice were adoptively transferred with bone marrow-derived DCs obtained from BALB/c mice. The obtained results demonstrated that intravenous injection of DCs returned the capacity of DCs-depleted CD11c-DTR mice to control the infection to the levels observed

in non-depleted animals clearly confirming that the observed increased severity of infection in DT-treated CD11c-DTR mice was specifically attributable to the reduction of DCs (Figure 16).

As the above-presented data indicates that DCs played an important role in host defense against *S. aureus* infection, it was then hypothesized that increasing DC numbers in normal BALB/c mice could improve their resistance to *S. aureus* infection. Indeed, adoptive transfer of either immature or LPS-matured DCs into normal BALB/ mice improved the capacity of these animals to clear *S. aureus* in the lungs (Figure 17). These results implied that DCs do not only play a protective role during *S. aureus* infection but may also be used as a therapeutic intervention.

The next step was to investigate the immunological mechanisms underlying the beneficial effect afforded by DCs during *S. aureus* bloodstream infection. As DCs are phagocytic cells, the ability of DCs to contribute to direct killing of *S. aureus* was evaluated. For this purpose, DCs were infected with *S. aureus* and the amount of intracellular bacteria was monitored at progressive times of infection. Interestingly, the DCs were not capable to kill the intracellular *S. aureus,* as the number of viable bacteria remained unaltered over time. Furthermore, live-imaging microscopy demonstrated the ability of *S. aureus* to replicate within the infected DCs (Video S2). Likewise, *S. aureus* has been shown to survive within other professional phagocytes, such as human [118] and murine PMNs [112] as well as human monocyte-derived macrophages [119]. Within these cells, *S. aureus* can evade the lethal effect of the phagolysosome by producing oxygen free radicals scavenger compounds such as catalases [120], staphyloxanthin [121], and yellow carotenoid pigments [12].

After the demonstration that DCs do not play a major role in direct bacterial clearance, it was then hypothesized that DCs may indirectly contribute to bacterial killing through their participation in the recruitment of inflammatory cells involved in bacterial eradication into the site of infection. As it has long been known that PMNs play a key role in the host innate immune response towards *S. aureus* [62-64], the amount of PMNs recruited in the lungs of DC-depleted and non-depleted mice during the course of *S.*

aureus infection was monitored. The obtained results revealed that the recruitment of PMNs in infected tissue was increased rather than decreased in mice depleted of DCs (Figure 20). These findings demonstrate that exacerbation of *S. aureus* infection in DC-depleted mice was not attributable to a reduced recruitment of PMNs and also suggest that increasing the number of PMNs in infected organs was rather detrimental to the host. Despite their role in bacterial clearance, there is now substantial evidence suggesting that through the release of cytotoxic compounds PMNs themselves can contribute to the pathogenesis of tissue injury associated with staphylococcal infection [122]. Furthermore, the results also indicate that the increase neutrophil numbers in DC-depleted mice correlate with increase in bacterial counts rather than with increased bacterial killing. Similar observations have been reported in murine models of *S. aureus* peritonitis [112] and hind paw infection [113] as well as in patients with *S. aureus* bloodstream infections [123-125].

Even more interestingly was the observation that neutrophils isolated from the lungs of DC-depleted mice harbored significantly higher numbers of intracellular viable *S. aureus* than those isolated from non-depleted animals (Figure 21). This implies that the ability of the recruited neutrophils to kill ingested *S. aureus* is even poorer in the absence of DCs. In this regard, several studies have provided evidence that local release of CXC chemokines promoted survival of *S. aureus* within neutrophils by facilitating bacterial internalization into an intracellular niche where they are protected from the host's killing mechanisms [112, 113]. In accordance with those studies, it was found that the levels of KC and MIP-2 in the lungs of DC-depleted *S. aureus*-infected mice were significantly higher than in non-depleted animals. Therefore, it is likely that DCs can regulate the production of CXC chemokine by other cell populations such as epithelial cells in the infected tissue.

In summary, the results of this study provide compelling evidence that DCs are important for host defense against *S. aureus* infection. It can be proposed that by orchestrating the local production of CXC chemokine and the recruitment and functional activity of PMNs DCs serve as modulators

of the local inflammatory responses elicited during *S. aureus* infection to prevent tissue injury.

DCs are also very important players during the development of an adaptive immune response [44]. $CD4^+$ T cells respond to peptide antigen displayed on MHC class II expressed on the surface of dendritic cells. Accessory molecules such as CD80 and CD86 expressed by DCs are required to ensure that T cells will divide and differentiate into effector cells. As DCs were capable to up-regulate the expression of MHC class II and accessory molecules in response to *S. aureus* infection, the capacity of infected DCs to stimulate naïve antigen-specific $CD4^+$ T cells was examined using OT-II transgenic mice. The results show that OVA-loaded *S. aureus*-infected DCs induced significantly higher levels of proliferation in $CD4^+$ T cells from OT-II mice than OVA-loaded uninfected DCs. These results indicate that DCs may also play an important role driving the generation of the primary antigen-specific immune response during *S. aureus* infection. More detailed analysis of the role of DCs on the acquired immune response to *S. aureus* should be performed in future studies.

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Appendix

A. Antibiotics and chemicals

[methyl-^{3}H]Thymidine	Amersham, Buchler, Germany
3,3´,5,5´-Tetramethylbenzidine (TMB)	Sigma Aldrich, Steinheim, Germany
Agarose	Sigma Aldrich, Steinheim, Germany
Ammonium chloride (NH_4Cl)	Roth, Karlsruhe, Germany
Aprotinin	Sigma Aldrich, Steinheim, Germany
Bovine Serum Albumin fraction V (BSA)	Applichem, Darmstadt, Germany
Carboxyfluorescein succinimidyl ester (CFSE)	eBiosciences, Frankfurt, Germany
Chloramphenicol	Sigma Aldrich, Steinheim, Germany
D-Glucose	Applichem, Darmstadt, Germany
Disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$)	Roth, Karlsruhe, Germany
DNase I	Quiagen, Hilden, Germany
Ethanol (96%)	J.T. Baker, Griesheim, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich, Steinheim, Germany
Fetal calf serum	Biochrom, Berlin, Germany
Gentamicin	Sigma Aldrich, Steinheim, Germany
Glycerol	Sigma Aldrich, Steinheim, Germany
Hydrochloric acid (HCL), 37%	J.T. Baker, Griesheim, Germany
Leupeptine	Merck, Darmstadt, Germany
Lysozyme	Sigma Aldrich, Steinheim, Germany
$Ova_{323-339}$ peptide	Genscript, Piscataway, NJ, USA

Phenylmethyl sulfonyl fluoride (PMSF)	Merck, Darmstadt, Germany
Polyoxyethylene Sorbitan Monolaurate (Tween-20)	Sigma Aldrich, Steinheim, Germany
Potassium bicarbonate ($KHCO_3$)	Roth, Karlsruhe, Germany
Potassium chloride (KCl)	Roth, Karlsruhe, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Roth, Karlsruhe, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Roth, Karlsruhe, Germany
Proteinase K	Merck, Darmstadt, Germany
RPMI 1620	Gibco-Invitrogen, Darmstadt, Germany
Sodium carbonate (Na_2CO_3)	Roth, Karlsruhe, Germany
Sodium chloride (NaCl)	Roth, Karlsruhe, Germany
Sodium hydroxide (NaOH)	Sigma Aldrich, Steinheim, Germany
Sulfuric acid (H_2SO_4)	Roth, Karlsruhe, Germany
Trizma Base	Sigma Aldrich, Steinheim, Germany

B. Buffer

0.05% Tween/PBS (5 l)	250 ml 20x PBS 2.5 ml Tween 20 *ad* 5 l *Aq. dest*.
1 M HCl (100 ml)	8.4 ml 37% HCl *ad* 100 ml *Aq. dest*.
1 M NaOH (500 ml)	20 g NaOH, *ad* 500 ml *Aq. dest*.

1x PBS, pH 7.4 (1 l)	50 ml 20x PBS, pH 7.4 *ad* 1 l *Aq. dest*., autoclave
20x PBS, pH 7.4 (5 l)	800 g NaCl 20 g KCl 144 g $Na_2HPO_4 \cdot 2H_2O$ 24 g KH_2PO_4 *ad* 5 l *Aq. dest*. adjust pH 7.4 with NaOH
5 mM Tris, pH 8.8 (100 ml)	0.0606 g Trizma Base, *ad* 100 ml *Aq. dest*. adjust pH 8.8 with HCl
Ammonium-Chloride-Potassium buffer (ACK)	0.01 M $KHCO_3$ 0.155 M NH_4Cl 0.1 mM EDTA *ad* 1 l *Aq. dest*. adjust pH 7.4 with HCl

Lysis buffer, pH 7.4 (1 l)	200 mM NaCl 5 mM ethylenediaminetetraacetic acid 10 mM Tris 10% glycerol 1 mM phenylmethyl sulfonyl fluoride 1 µg/ml leupeptine 28 µg/ml aprotinin *ad* 1 l *Aq. dest*. adjust pH 7.4 with NaOH, store at -20 °C

C. Expendable materials and Instruments

Blood agar plates	Invitrogen, Darmstadt, Germany
Cell culture plates	Invitrogen, Darmstadt, Germany
Cell harvester	Inotech, Wohlen, Switzerland
Cell nylon meshes	BD Bioscience, NY, USA
Centrifuge Tubes	Greiner Bio One, Frickenhausen, Germany
ELISA Reader	Tecan Group Ltd, Männedorf, Switzerland
FACSCalibur™ flow cytometer	BD Biosciences, CA, USA
Filtermats A 1450-421	Perkin Elmer/ Wallac, Freiburg, Germany
Heraeus Biofuge	Thermo Electron, Bremen, Germany

Manual MACS Separators	Miltenyi Biotec, Bergisch Gladbach, Germany
MeltiLex solid scintillator	Perkin Elmer/ Wallac, Freiburg, Germany
MicroBeta cassette	Perkin Elmer/ Wallac, Freiburg, Germany
Microcentrifuge tubes	Eppendorf, Hamburg, Germany
MS columns	Miltenyi Biotec, Bergisch Gladbach, Germany
Needles	Braun, Melsungen, Germany
Pipetboy	IBS Integra Bioscience, Fernwald, Germany
Pipette, 5ml and 10ml	Greiner Bio One, Frickenhausen, Germany
Syringes	Braun, Melsungen, Germany
Thermomixer 5436	Eppendorf, Hamburg, Germany
Tissue homogenizer	Biolab, Gödenstorf, Germany
Vortex-Genie2	Scientific Industries, NY, USA
Wallac MicroBeta® TriLux	Perkin Elmer/ Wallac, Freiburg, Germany

D. Media for bacteria culture

Brain heart infusion (BHI)	Sigma Aldrich, Steinheim, Germany 37g BHI culture media, *ad* 1 l *Aq. dest*., autoclave

E. Media and supplements for cell culture

Fetal calf serum (500 ml)	Adjust 500 ml FCS to 37 °C ,heat-inactivate for 30 min at 56 °C, store at -20 °C
L-Glutamine (200 mM)	PAA Laboratories, Pasching, Austria
Penicillin/Streptomycin, 100x	PAA Laboratories, Pasching, Austria
Recombinant murine GM-CSF	eBiosciences, Frankfurt, Germany
Recombinant murine IL-4	eBiosciences, Frankfurt, Germany
RPMI complete medium (500 ml)	RPMI 1620, add 10% FCS 100 U/ml Penicillin 50 mg/ml Streptavidin 1 mM L-Glutamine 0.5 µM 2-mercaptoethanol

F. Antibodies

Anti-CD11c magnetic beads	Miltenyi Biotec, Bergisch Gladbach, Germany
Anti-CD40	Invitrogen, Darmstadt, Germany
Anti-CD4-PE	BD Bioscience, NY, USA
Anti-CD80	Invitrogen, Darmstadt, Germany
Anti-CD86	Invitrogen, Darmstadt, Germany
Anti-MHC class II	Invitrogen, Darmstadt, Germany
Anti-PE magnetic beads	Miltenyi Biotec, Bergisch Gladbach, Germany

Anti-RB6	Kind gift from Dr. Siegfried Weiß, HZI
BD OptEIA^{TM} Mouse ELISA kit (IL-6, IL-12, TNF-α)	eBiosciences, Frankfurt, Germany
KC and MIP ELISA kit	R&D Systems, Minneapolis, USA

G. Mice

B6.FVB-Tg [Itgax-DTR/GFP] 57Lan/J transgenic mice (CD11c-DTR)	Kind gift from Steffen Jung, The Weizmann Institute of Science, Rehovot, Israel
BALB/c	Harlan-Winkelmann, Borchen, Germany
Ovalbumin specific mice B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-II)	Jackson Laboratory, Maine, USA

H. Abbreviations

°C	degree Celsius
µg	Microgram
BHI	Brain-heart infusion
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl ester
CFU	colony forming units
DC	dendritic cell
dH_2O	distilled water
DT	diphtheria toxin
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FCS	fetal calf serum

FnBPA	fibronectin binding protein
GFP	green fluorescencent protein
h	Hour
H&E	hematoxylin and eosin
i.p.	Intraperitoneal
i.v.	Intravenous
LPS	lipopolysaccaride
MHC	major histocompatibility complex
min	Minute
ml	Milliliter
mM	milli molar
MOI	multiplicity of infection
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMMs	microbial surface components recognizing adhesive matrix molecules
NADPH	nicotinamide adenine dinucleotide phosphate
nm	Nanometer
PBS	phosphate buffered saline
pH	*potentia Hydrogenii*
PMN	polymorphonuclear PMNs
Rpm	rotates per minute
S. aureus	*Staphylococcus aureus*
SD	standard deviation
TCR	T cell receptor
TMB	3,3',5,5'-Tetramethyl benzidine
TSST-1	toxic shock syndrome toxin 1
VRSA	Vancomycin-resistant *Staphylococcus aureus*
YOPs	*Yersinia* outer proteins

I. Videos

The Videos S1 (Phagocytic uptake of *S. aureus* by DCs) and S2 (*S. aureus* replicates within the DC) are available as avi files. Printed copies of this dissertation comprise the videos on the enclosed CD-R.

J. List of figures